(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 May 2001 (31.05.2001)

PCT

(10) International Publication Number
WO 01/38579 A2

(51) International Patent Classification[7]: C12Q 1/68, C12N 5/10, 15/63, C07K 14/47, 16/18, C07H 21/04

(21) International Application Number: PCT/US00/32049

(22) International Filing Date:
21 November 2000 (21.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 60/166,923 | 22 November 1999 (22.11.1999) | US |
| 60/183,531 | 18 February 2000 (18.02.2000) | US |
| 09/717,321 | 20 November 2000 (20.11.2000) | US |

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

| | |
|---|---|
| US | 60/166,923 (CIP) |
| Filed on | 22 November 1999 (22.11.1999) |
| US | 60/183,531 (CIP) |
| Filed on | 18 February 2000 (18.02.2000) |
| US | 09/717,321 (CIP) |
| Filed on | 20 November 2000 (20.11.2000) |

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *Without international search report and to be republished upon receipt of that report.*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/38579 A2

(54) Title: METHOD OF IDENTIFYING TOXIC AGENTS USING NSAID-INDUCED DIFFERENTIAL GENE EXPRESSION IN LIVER

(57) Abstract: The invention provides methods of identifying toxic agents, e.g., hepatotoxic agents, using differential gene expression. Also provided are methods of predicting the risk level and or injury type of NSAIDs. Also disclosed are novel nucleic acid sequences whose expression is differentially regulated by NSAIDs.

# METHOD OF IDENTIFYING TOXIC AGENTS USING NSAID-INDUCED DIFFERENTIAL GENE EXPRESSION IN LIVER

## FIELD OF THE INVENTION

The invention relates generally to nucleic acids and polypeptides, and more particularly to the identification of differentially expressed nucleic acids and proteins in liver.

## BACKGROUND OF THE INVENTION

Liver is the primary organ for biotransformation of chemical compounds and their detoxification. Liver injury produced by chemicals has been recognized for over 100 years, and hepatic damage is one of the most common toxicities among drugs at pre-clinical and clinical stages of drug development. Over 30% of new chemical entities (NCE) are generally terminated due to adverse liver effects in humans. During a period of 30 years, hepatotoxicity has been the major cause of drug withdrawal for safety reasons at the marketing stage, accounting for 18% overall drug withdrawal. Many of the drugs that are withdrawn from market due to hepatotoxicity produce lethality in a small percentage of patient population and are classified as type II lesion (or idiosyncratic, sporadic) toxicity.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a group of unrelated chemical compounds that have been used to successfully treat rheumatic and musculospastic disease. Unfortunately, unwanted hepatotoxic side effects have lead to the premature market withdrawal of several NSAIDs, including Cincophen, Benoxaprofen, Piroxicam, Suprofen, and Bromfenac. The pervasiveness of idiosyncratic reactions of many NSAIDs has lead the Food and Drug Administrations Arthritis Advisory Board to conclude that NSAIDs as a group should be considered to induce hepatotoxicity.

It is estimated that annual NSAIDS consumption in the U.S exceeds 10,000 tons. Due to this large consumption of NSAIDS for a wide variety of pain and inflammatory conditions, it has become an important class of drugs responsible for liver injury, despite the overall extremely low incidence of producing hepato-toxicity. Liver injury resulting from NSAIDs can have several forms, including acute toxicity resulting from hepatocellular (parenchymal) damage (*e.g.* necrosis) and arrested bile flow (cholestasis). Thee general mechanism that is thought to mediate NSAIDS toxicity is idisyncratic reaction (Type II) to the drug (both immunologic and metabolic), which is dose independent, and presumably results from interindividual variation in drug metabolism. Currently no clear mechanism of drug-induced

idiosyncratic toxicity is available. Accordingly, there remains a great need to elucidate the molecular basis of idiosyncratic hepatoxicity, such as NSAID-induced toxicity, including the identification of genes and proteins differentially expressed in response to administration of such drugs.

## SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided methods of screening and identifying test agents which induce hepatotoxicity, *e.g.* idiosyncratic hepatotoxicity. The methods of the invention are based in part on the discovery that certain nucleic acids are differentially expressed in liver tissue of animals treated with NSAIDs. These differentially expressed nucleic acids include novel sequences that, while previously described, have not heretofore been identified as responsive to drugs, such as NSAIDs, which induce idiosyncratic hepatoxicity.

In various aspects, the invention includes a method of screening a test agent for toxicity, e.g., idiosyncratic hepatotoxicity. For example, in one aspect, the invention provides a method of identifying a hepatotoxic agent by providing a test cell population comprising a cell capable of expressing one or more nucleic acids sequences responsive to drugs, *e.g.* NSAIDs, which induce idiosyncratic hepatotoxicity, contacting the test cell population with the test agent and comparing the expression of the nucleic acids sequences in the test cell population to the expression of the nucleic acids sequences in a reference cell population. An alteration in expression of the nucleic acids sequences in the test cell population compared to the expression of the gene in the reference cell population indicates that the test agent is hepatotoxic. In one aspect, expression in the test cell population is compared to the expression of a reference cell population exposed to a NSAID that is classified as low risk, very low risk, or overdose risk of hepatoxicity, thereby to predict whether the test agent has low, very low, or overdose risk of hepatoxicity. In another aspect, the test cell population is compared to the expression of a reference cell population exposed to a NSAID which induces a known type of hepatic injury, *e.g.* hepatocellular damage, cholestasis, or elevated transaminase level, thereby to predict whether the test agent is likely to induce a given type of hepatoxic injury.

In a further aspect, the invention provides a method of assessing the hepatotoxicity, *e.g.* idiosyncratic hepatotoxicity, of a test agent in a subject. The method includes providing from the subject a cell population comprising a cell capable of expressing one or more NSAID-responsive genes, and comparing the expression of the nucleic acids sequences to the

expression of the nucleic acids sequences in a reference cell population that includes cells from a subject whose exposure status to a hepatotoxic agent is known. An alteration in expression of the in the test cell population compared to the expression of the nucleic acids sequences in the reference cell population indicates the hepatotoxicity of the test agent in the subject.

Also provided are novel nucleic acids, as well as their encoded polypeptides, whose expression is responsive to the effects of NSAIDS.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

## DETAILED DESCRIPTION OF THE INVENTION

The invention is based in part on the discovery of nucleic acid sequences which are differentially expressed in rodent liver cells upon administration of NSAIDS. The discovery includes groups of nucleic acid sequences whose expression is correlated with hepatotoxicity risk associated with, and injury type induced by, NSAID administration.

The differentially expressed nucleic acid sequences were identified by examining 29 different NSAIDS that have varying degrees of hepatotoxicity. These 29 drugs, shown in Table 1, below, were first categorized as low dose (1-75 mg/kg) and high dose (above 75 mg/kg) drugs, then classified as non-toxic, toxic, and those withdrawn from market (within each dose). Each of the 29 NSAIDS was administered orally to groups (3 animals per group) of 12 week old male Sprague Dawley rats for 72 hours (3 days) at the dosages specified in Table 1 (e.g. Naproxen: 54 mg/kg/day PO in QD x 3 days in $H_2O$). Vehicle controls (water, ethanol, canola oil) were also included (3 animals per group). The animals were sacrificed 24 hours after the final dose, liver tissue was removed on necroscopy, and total RNA was recovered from the dissected tissue. Complementary DNA (cDNA) was prepared and samples were processed through GENECALLING™ differential expression analysis, as described in U.S. Patent No. 5,871,697 and in Shimkets *et al.*, *Nature Biotechnology 17:* 798-803 (1999), the disclosures of which are hereby incorporated by reference herein.

Table 1: NSAIDS and Dosages Administered

| Compound | Dose | Vehicle |
|---|---|---|
| *Acetaminophen* | 133 mg/kg/day p.o. in QD x 3 days. | 10 %Ethanol Vehicle |
| *Acetylsalicylic Acid* | 200 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Benoxaprofen* | 16 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Bromfenac* | 7.5 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Celecoxib* | 89 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Diclofenac* | 38 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Etodolac* | 30 mg/kg/day p.o. in QD x 3 days. | 10% Ethanol Vehicle |
| *Felbinac* | 33 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Fenoprofen* | 154 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Flurbiprofen* | 10 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Ibuprofen* | 211 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Indomethacin* | 4 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Ketoprofen* | 10 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Ketorolac* | 1.5 mg/kg/day p.o. in QD x 3 days. | 10% Ethanol Vehicle |
| *Meclofenamate* | 20 mg/kg/day p.o.in QD x 3 days. | H2O Vehicle |
| *Mefenamic Acid* | 79 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Nabumetone* | 143 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Naproxen* | 54 mg/kg/day p.o. in QD x 3 days. | 10% Ethanol Vehicle |
| *Olsalazine* | 222 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Oxaprozin* | 100 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Phenacetin* | 100 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Phenylbutazone* | 100 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Piroxicam* | 20 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Sulindac* | 77 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Sulphasalazine* | 338 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Suprofen* | 20 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Tenoxicam* | 10 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |
| *Tolmentin* | 100 mg/kg/day p.o. in QD x 3 days. | H2O Vehicle |
| *Zomepirac* | 19 mg/kg/day p.o. in QD x 3 days. | Canola Oil Vehicle |

3635 gene fragments were initially found to be differentially expressed in rat liver tissue (analysis of variance, $p<0.01$) in response to these compounds. The compounds were then classifed according to hepatotoxicity risk, as indicated in Table 2.

Table 2: Hepatotoxicity Risk of NSAIDs

| Compound | Risk |
|---|---|
| Acetaminophen | Overdose Risk |
| Acetylsalicylic Acid | Overdose Risk |
| Benoxaprofen | Low Risk |
| Bromfenac | Low Risk |
| Celecoxib | Unknown |
| Diclofenac | Low Risk |
| Etodolac | Very Low Risk |
| Felbinac | Unknown |
| Fenoprofen | Very Low Risk |
| Flurbiprofen | Very Low Risk |
| Ibuprofen | Very Low Risk |
| Indomethacin | Very Low Risk |
| Ketoprofen | Very Low Risk |
| Ketorolac | Unknown |
| Meclofenamate | Very Low Risk |
| Mefenamic Acid | Very Low Risk |
| Nabumetone | Very Low Risk |
| Naproxen | Very Low Risk |
| Olsalazine | Unknown |
| Oxaprozin | Very Low Risk |
| Phenacetin | Overdose Risk |
| Phenylbutazone | Low Risk |
| Piroxicam | Very Low Risk |
| Sulindac | Low Risk |
| Sulphasalazine | Unknown |
| Suprofen | Very Low Risk |
| Tenoxicam | Very Low Risk |
| Tolmentin | Very Low Risk |
| Zomepirac | Very Low Risk |

In order to discriminate among these groups, the above compound set was divided into a training set (consisting of three compounds per group), and a test set (consisting of the remainder. This was done to minimize the reliance on the assumptions required for parametric analyses. Compounds with unknown risk were not used in this analysis. The training set employed is shown in Table 3.

Table 3: Training Set of NSAIDs by Risk Classification

| Control | Low Risk | Very Low Risk | Overdose Risk |
|---|---|---|---|
| Sterile water | Benoxaprofen | Flurbiprofen | Acetaminophen |
| 10% Ethanol | Phenylbutazone | Oxaprozin | Acetylsalicylic Acid |
| Canola oil | Sulindac | Tenoxicam | Phenacetin |

The 3635 differentially expressed nucleic acid fragments were then analyzed using a stepwise multivariate analysis of variance as follows:

1. Calculate 3635 T2 (yi1) (Hoettelling's trace, one of the test statistics used for this analysis) values, one for each differentially expressed fragment. The fragment with the largest individual T2 value is selected as the first discriminatory set (yi1).
2. Calculate 3634 T2 (yi1,yi2) values, one for each combination of two fragments. The fragment pair with the largest individual T2 value are selcted as the second discriminatory set.
3. Calculate 3626 T2 (yi1,yi2,yi3,...yi,10), one for each combination of ten fragments. The fragment set with the largest T2 value are selected as the final discriminatory set.

This stepwise procedure is used whenever the number of dependent variables (gene fragments) exceeds the number of independent variables (samples). In addition to fragment addition, fragment elimination occurs whenever an added fragment no longer contributes significant discriminatory power to the existing set. This eliminates bias as to the order fragments enter the model (Ahrens and Läuter, Mehrdimensionale Varianzanalyse, Akademie-Verlag, Berlin (1974); Dziuda, *Medical Inform. 15(4):* 319 (1990)).

This analysis protocol identified ten fragments that significantly ($p=6.02 \times 10^{-28}$) discriminated among the drugs in the test set. Two fragments on this list were not required to maintain the discriminatory ability and were subsequently removed ($p=3.96 \times 10^{-26}$). Differential expression of these gene fragments were successfully confirmed using an unlabeled oligonucleotide competition assay (Shimkets *et al.*, *Nature Biotechnology 17:* 198-803 (1999)). The 8 fragments (RISKMARKER 1-8) represent both novel and known rat genes for which the sequence identity to genes in public databases is either high (>90%), moderate (70-90%), or low (<70%).

The identity of these 8 hepatoxcitiy risk discriminatory nucleic acid sequences(with GenBank accession numbers) are further described below. Where appropriate, the cloned sequence from isolation is provided; this sequence was then extended using either Genbank rat ESTs or from internally (Curagen Corporation) sequenced rat fragments. The extended contig sequence is provided as "consensus." Finally, the best BlastN and BlastX results are also provided. In some instances the cloned sequence is identical to a known rat gene, in those

instances the name of the gene and a database accession number and the sequence listed in the database is provided:

**RISKMARKER 1**

RISKMARKER 1 is a novel 1265 bp gene fragment , which has 67% sequence identity to human rac1 genomic fragment [AJ132695], probable 3' UTR. The nucleic acid was initially identified in a cloned fragment having the following sequence:

```
1   caattgaaaa aagtttgttc tagtggtcga aaggcccaac actgtgttct tgccagtgag
61  ttaggttgta cagaacggcg ttagcactag cgcttgacag aacctcacag acccaaaggt
121 acc  (SEQ ID NO: 1)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
   1 TTTTTTTTTTTTTTTTTCAAGTTCCAAAGACATTTTTTTTTTTTTTTTTATGATTCAAGGATTTATTAAGTCATACATGC
  81 AAAACATACTGCTAACTGCATTAGCAAAAGATCAATGTAAAAACACTCCACAATTCTGCAACTGTCAATTGAAAAAAGTT
 161 TGTTCTAGTGGTCGAAAGGCCCAACACTGTGTTCTTGCCAGTGAGTTAGGTTGTACAGAACGGCGTTAGCACTAGCGCTT
 241 GACAGAACCTCACAGACCCAAAGGTACCGGAAGCATGTGTCCGCGTGGGTGAGGTCTAGAGGGGGCGGCATCAATCACAT
 321 GACAGTGTTGGTACTCTGGCAAGACAGTGATGTTTCAGAATATCTAAAATAGTTTAAAAACTGTAAAGCCGCAGCACGTG
 401 ATTTCTACACCCAGTTACTAGAAAACGAAGGGAAGCACTAGTCAGCTGAGTAAAGGAAGGTGAAAACAGGAACGCACTTC
 481 TACTATCTACCAAAAAAATCTCCGAATGCATTATCAGAAAGATCTTATAGTACAGGTCAGACATATTGCTCGTTAAGAAG
 561 GGGGTCCTAAAGAAAAGCACTTGCTAAGTTAGCAACTGTGAGGATGGCCAGTTTAAATATGGACTCAACGCCCCATCTGG
 641 GGAGGGACAGCAGGGGGAAGGGGGGCTCAAGAGAGACACTGATAAGATCGGCCATTTGTCATCTACTGTTTGACAGAAAT
 721 TAACCGTTAAAAAGCTTTACCCGTGACACTTTTATTCAGTTGAATTACTCCATGTACAATGTAGTGTAAATTAATCTCTA
 801 CTTCATATTAGTCAAAATACTGTCTGTCTCCTTTGATGACGTCGTGTTTCACACACTCCACCCAGCACACCCACGACTAG
 881 GAACAGAATACTTCGTTAGAGGCAACACAGGAGCCAGAGTTCTGTTCAAAGCCTGCAGAAGCCGGTCAGCTGGTATTTTA
 961 GAGAACTCACTATGAAATCAAAGAGCAGAGCTGTTACACCCATCGTGACGTACAGTACAAAGTTACGTAATGAGCATGGG
1041 CTGATAAGTTACAGGTGCGTTACATGGCAGCGTGTCATTAAGGAGGCTGTGCTGTGTCACACGGTCTGGGAGCTACGGGA
1121 GGGTCTGCACCCCTGAGCCCAGAAGCTGCAGTCTTCTTAAGGACAAAGTCTCTCAACAGCTTAGTGCTTACGTGTTCTCA
     GCACAACGCAACTTAGTTCACAAGGTATTTTGGCAATTCTTAATCTGAGCAAGAATAGGGGATTTT
1201
     (SEQ ID NO: 2)
```

Blast-N Results:

**>gb:GENBANK-ID:HSA132695|acc:AJ132695 Homo sapiens rac1 gene - Homo sapiens, 28567 bp.**

Top Previous Match Next Match
Length = 28,567

Minus Strand HSPs:

```
 Score = 1328 (199.3 bits), Expect = 2.8e-90, Sum P(2) = 2.8e-90
 Identities = 694/1022 (67%), Positives = 694/1022 (67%), Strand = Minus / Plus

Query:  1261 CCCCTATTCTTGCTCAGATTAAGAATTGCCAAAATACCTTGTGAACTAAGTTGC---GTT 1205
             |||| ||||||| |||||||||| |||||||||||||| |||||||   |||   |||
Sbjct: 26871 CCCCCATTCTTGTTCAGATTAAGAGTTGCCAAAATACCTTCTGAACTACACTGCATTGTT 26930

Query:  1204 GTGCTGAGAACACGTAAGCACTAAGCTGTTG-AGAGACTTT-GTCCTTAAGAAGACTGCA 1147
             |||| ||||||||  | ||||| | || ||| | |||| || ||| || ||||||| | |
Sbjct: 26931 GTGCCGAGAACACCGA-GCACTGAACT-TTGCAAAGACCTTCGTCTTTGAGAAGACGGTA 26988

Query:  1146 GCTTCTGGGCTCAGG-GGTGCAGACCCTCCCGTAGCTCCCAGACCGTGTGACACAGCACA 1088
             ||||||||   | ||| |||||||||| ||  | |  |||| |    || |  | ||| |
Sbjct: 26989 GCTTCTGCAGTTAGGAGGTGCAGACACTTGC-T--CTCCTATGTAGT-T--CTCAG-AT- 27040
```

```
Query:  1087 GCCTCCTTAATGACACGCTGCC--ATGTAACGCA-CCTGT-AACTTATCAGCCCAT--GC 1034
Sbjct: 27041 GCGTAAAGCAGAACAGCCTCCCGAATGAAGCGTTGCCATTGAACTCACCAGTGAGTTAGC 27100

Query:  1033 T-CA--T-TACGTAACTTTGTACTGTAC-GTCACGATGG-GTGTAACAGCTCTGCTCTTT 980
Sbjct: 27101 AGCACGTGTTCCCGACATAACATTGTACTGT-A--ATGGAGTG-AGCGTAGCAGCTCAGC 27156

Query:   979 GATTTCATAGTGAGT-TCTCTAAAATACCAGCTGACCGGCTTCTG-CAGGCTTT-GAACA 923
Sbjct: 27157 TCTTTGG-A-TCAGTCTTTGTGATTTCATAGC-GA--GTTTTCTGACCAGCTTTTGCGGA 27211

Query:   922 GAACTCTGG-CTC--CTG-TGTTGCCTCTAACGAAGTATTCTGTTCCTAGTCGTGGGTGT 867
Sbjct: 27212 GATTT-TGAACAGAACTGCTATTTCCTCTAATGAAGAATTCTGTT--TAGCTGTGGGTGT 27268

Query:   866 GCTGGGTGGAGTGTGTGAAACACGACGTCATCAAAGGAGACAGACAGTATTTTGACTAA- 808
Sbjct: 27269 GCCGGGTGGGGTGTGTGT-----GA-----TCAAAGGACAAAGACAGTATTTTGACAAAA 27318

Query:   807 TATGAAGTAGAGATTAATT-TACACT--ACATTGTACATGGAGTAAT-TCAACTGAATAA 752
Sbjct: 27319 TACGAAGTGGAGATTTACACTACATTGTACAAGG-A-ATGAA--AGTGTCACGGGTA-AA 27373

Query:   751 AAGTGTCACGGGTAAAGCTTTTT-AACGGTTAATTTCTG-TCAAACAGTAGATGACAA-A 695
Sbjct: 27374 AACTCTAAAAGGTTAATTTCTGTCAAATGC-AGTAGATGATGAAAGAAAGGTTGGTATTA 27432

Query:   694 TG-GCCGATCTTATCAGTGTCTCTCTTGAGCCCCCCTTCCCCCTGCTGTCCCTCCCCAGA 636
Sbjct: 27433 TCAGGAAATGTTTTCTTAAGCT-T-TTCCTTTCTC-TTACACCTGCCATGCCTCCCCAAA 27489

Query:   635 TGGGGCGTTGAGTCCATATTTAAACTGGCCATCCTCACAGTTGCTAACTTAGCAAGTGCT 576
Sbjct: 27490 TTGGGCATTTAATTCATCTTTAAACTGGTTGTTCTGTTAGTCGCTAACTTAGTAAGTGCT 27549

Query:   575 TTTCTT-TAGGACCCCCTTCTTAACGAGCAATATGTCTGACCTGTACTATAAGATCTTTC 517
Sbjct: 27550 TTTCTTATAGAACCCC-TTCTGACTGAGCAATATGCCTC-CTTGTATTATAAAATCTTTC 27607

Query:   516 TGATAATGCATTCGGAGATTTTTTTGGTAGATAGTAGAAGTGCGTTCC-TGTTTTCACCT 458
Sbjct: 27608 TGATAATGCATTAGAAGGTTTTTTTGTCGATTAGTAAAAGTGCTTTCCATGTTACTTTAT 27667

Query:   457 TCCTT--TACTCAG--CTGAC-T-AGTGCTTCCCTTCGTTT--TCTAGTAA-C-TGGGT- 409
Sbjct: 27668 TCAGAGCTAATAAGTGCTTTCCTTAGTT-TTCTAGTAACTAGGTGTAAAAATCATGTGTT 27726

Query:   408 GTAGAAATCACGTGCTGCGGCT-TTACAG-T--TT-TTAAACTATTTTAGATATTCTGAA 354
Sbjct: 27727 GCAGCTTTATAGTTTTTAAAATATTTTAGATAATTCTTAAACTATG--A-ACCTTCTTAA 27783

Query:   353 ACATCACTGTCTTGCCAGAGTACCAACACTGTCATGTGATTGATGCCGCCCCCTCTAGAC 294
Sbjct: 27784 -CATCACTGTCTTGCCAGATTACCGACACTGTCACTTGACCAATACTGACCC-TCTTTAC 27841

Query:   293 CTCACCCACGCGGACACATGCTTCCGGTA--C-CTTTGGGTCTGTGAGGTTC 245
Sbjct: 27842 CTCGCCCACGCGGACACACGCCTCCTGTAGTCGCTTTGCCTAT-TGATGTTC 27892

 Score = 930 (139.5 bits), Expect = 2.8e-90, Sum P(2) = 2.8e-90
 Identities = 270/354 (76%), Positives = 270/354 (76%), Strand = Minus / Plus

Query:   357 TGAAACATCACTGTCT-TGCCAGAGTACCAACACTGTCATGTGATTGATGCCGCCCCCTC 299
Sbjct: 27795 TGCCAGATTACCGACACTGTCACTTGACCAATACTGACCC-TCTTT-ACCTCGCCCACGC 27852

Query:   298 TAGAC-CTCACCCAC-GCGGACACAT-GCTTCC-G--GTACCTTTGGGTCTGTGAGGTTC 245
Sbjct: 27853 G-GACACCGCCTCCT GT|GTCGCTTTGCCTATTGATGTTCCTTTGGGTCTGTGAGGTTC 27911

Query:   244 TGTCAA--GCGCTAGTGCTAACGCCGTTCTGTACAACCTAACTCACTGGCAAGAACACAG 187
Sbjct: 27912 TGTAAACTGTGCTAGTGCTGACGATGTTCTGTACAACTTAACTCACTGGCGAGAATACAG 27971

Query:   186 TGTTGGGCCTTTCGACCACTAGAACAAACTTTTTTCAATTGACAGTTGCAGAATTGTGGA 127
Sbjct: 27972 CGTGGGACCCTTCAGCCACTACAACAGAATTTTTTAAATTGACAGTTGCAGAATTGTGGA 28031
```

```
Query:   126 GTGTTTTTACATTGATCTTTTGCTAATGCAGTTAGCAGTATGTTTTGCATGTATGACTTA 67
             GTGTTTTTA|ATTGAT|TTTTG|TAATG|A TTAG|A TATGTTTTG|ATGTATGA|TTA
Sbjct: 28032 GTGTTTTTACATTGATCTTTTGCTAATGCAATTAGCATTATGTTTTGCATGTATGACTTA 28091

Query:    66 ATAAATCCTTGAATCATAAAAAAAAAAAAAAAAAATGTCTTTGGAACTTGAAAAAAAA 10
             ATAAAT||TTGAAT|ATA  A     AA A    TGT TTTG  A|TTGA  AA AA
Sbjct: 28092 ATAAATCCTTGAATCATACGACTGGTAATACTGGTGTTTTTGAGACTTGATGAACAA 28148
```

## RISKMARKER2

RISKMARKER2 is a 650 bp rat expressed sequence tag (EST) [AW435096]. The nucleic acid sequence was initially identified in a cloned fragment having the following sequence:

```
  1 TTTTTTTTTTTTTTTTTGGCAGAATTCTGATGTTTACTGGGACCCATAGTAGTCAAGGTGACAGCAAGGGTAGGGGAGGA
 81 AACTCAGCAGAGGCGGATCCCAGGTCTGGAGGGAAGCTGACAGCAGCCCAGTAAGCTGTGCCAGAAGGCTGTAACAGTAG
161 CGGAGCCAGTGACAGCGCCAGGCTGGGCTGGGTTCTCTCTGTGGGTGTGCACGGCAAAGCTGCGGCCTGTGGGCCCTGGG
241 GGGCCTGTCAGCTCCACATCCACCACATGCATGTCGGTGAGGCTAAGGTCAGCCACAAGCACCCCAATGACACGATCAAA
321 GCCTAGACTGGGAGCGGCCAGGGCAGCGGCTGCCATGGTGTTGGAGTTTCGGGGGGCCAAGGGGCAGAGCCCACGCACAG
401 GGCCCTCATAGAGCACTGTGCGGGGCCCACTACTATGTGCGGCAGCCAGGGGTCNCTCCAGCCGGAAGCCATCAGGATGT
481 GTGG (SEQ ID NO: 3)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
  1 TTTTTTTTTTTTTTTTTGGCAGAATTCTGATGTTTACTGGGACCCATAGTAGTCAAGGTGACAGCAAGGGTAGGGGAGGA
 81 AACTCAGCAGAGGCGGATCCCAGGTCTGGAGGGAAGCTGACAGCAGCCCAGTAAGCTGTGCCAGAAGGCTGTAACAGTAG
161 CGGAGCCAGTGACAGCGCCAGGCTGGGCTGGGTTCTCTCTGTGGGTGTGCACGGCAAAGCTGCGGCCTGTGGGCCCTGGG
241 GGGCCTGTCAGCTCCACATCCACCACATGCATGTCGGTGAGGCTAAGGTCAGCCACAAGCACCCCAATGACACGATCAAA
321 GCCTAGACTGGGAGCGGCCAGGGCAGCGGCTGCCATGGTGTTGGAGTTTCGGGGGGCCAAGGGGCAGAGCCCACGCACAG
401 GGCCCTCATAGAGCACTGTGCGGGGCCCACTACTATGTGCGGCAGCCAGGGGTCCCTCCAGCCGGAAGCCATCAGGATGT
481 GTGGCCATGGTGACTCGAAGGCTCTGGAGGCCTCCGGCTGCATCCAATCTGCTGATGTCTTCACAACCCCACAGGGCCCC
561 TCGGGCCACAAACACCGTGTGGCCCCAGTGGTTTGAAGCCTCCAGGAGCTGCCGCTCTGTGGTCTGGTCAGCGAGAGCTG
641 AGGGGGATCC  (SEQ ID NO: 4)
```

Blast-N Results:

```
>gb:GENBANK-ID:AW435096|acc:AW435096 UI-R-BJ0p-afy-e-10-0-UI.s1 UI-R-BJ0p
Rattus norvegicus cDNA clone UI-R-BJ0p-afy-e-10-0-UI 3', mRNA sequence -
Rattus norvegicus, 484 bp (RNA).
          Length = 484

  Plus Strand HSPs:

 Score = 2413 (362.0 bits), Expect = 1.2e-102, P = 1.2e-102
 Identities = 483/484 (99%), Positives = 483/484 (99%), Strand = Plus / Plus

Query:     1 TTTTTTTTTTTTTTTTTGGCAGAATTCTGATGTTTACTGGGACCCATAGTAGTCAAGGTG 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 TTTTTTTTTTTTTTTTTGGCAGAATTCTGATGTTTACTGGGACCCATAGTAGTCAAGGTG 60
```

```
Query:   61 ACAGCAAGGGTAGGGGAGGAAACTCAGCAGAGGCGGATCCCAGGTCTGGAGGGAAGCTGA 120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   61 ACAGCAAGGGTAGGGGAGGAAACTCAGCAGAGGCGGATCCCAGGTCTGGAGGGAAGCTGA 120

Query:  121 CAGCAGCCCAGTAAGCTGTGCCAGAAGGCTGTAACAGTAGCGGAGCCAGTGACAGCGCCA 180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  121 CAGCAGCCCAGTAAGCTGTGCCAGAAGGCTGTAACAGTAGCGGAGCCAGTGACAGCGCCA 180

Query:  181 GGCTGGGCTGGGTTCTCTCTGTGGGTGTGCACGGCAAAGCTGCGGCCTGTGGGCCCTGGG 240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  181 GGCTGGGCTGGGTTCTCTCTGTGGGTGTGCACGGCAAAGCTGCGGCCTGTGGGCCCTGGG 240

Query:  241 GGGCCTGTCAGCTCCACATCCACCACATGCATGTCGGTGAGGCTAAGGTCAGCCACAAGC 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  241 GGGCCTGTCAGCTCCACATCCACCACATGCATGTCGGTGAGGCTAAGGTCAGCCACAAGC 300

Query:  301 ACCCCAATGACACGATCAAAGCCTAGACTGGGAGCGGCCAGGGCAGCGGCTGCCATGGTG 360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  301 ACCCCAATGACACGATCAAAGCCTAGACTGGGAGCGGCCAGGGCAGCGGCTGCCATGGTG 360

Query:  361 TTGGAGTTTCGGGGGGCCAAGGGGCAGAGCCCACGCACAGGGCCCTCATAGAGCACTGTG 420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  361 TTGGAGTTTCGGGGGGCCAAGGGGCAGAGCCCACGCACAGGGCCCTCATAGAGCACTGTG 420

Query:  421 CGGGGCCCACTACTATGTGCGGCAGCCAGGGGTCCCTCCAGCCGGAAGCCATCAGGATGT 480
            |||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||
Sbjct:  421 CGGGGCCCACTACTATGTGCGGCAGCCAGGGGTCNCTCCAGCCGGAAGCCATCAGGATGT 480

Query:  481 GTGG 484
            ||||
Sbjct:  481 GTGG 484
```

Blast-X Results:

**>ptnr:SPTREMBL-ACC:Q19527 F17C8.3 PROTEIN - Caenorhabditis elegans, 973 aa.**

Top Previous Match Next Match

Length = 973

Minus Strand HSPs:

Score = 351 (123.6 bits), Expect = 6.3e-30, P = 6.3e-30
Identities = 78/161 (48%), Positives = 96/161 (59%), Frame = -1

```
Query: 650 GSPSALADQTTERQLLEASNHWGHTVFVARGALWGCEDISRLDAAGGLQSLRVTMATHPD 471
       GSP+ A+Q  +L+S  G++ GALWG DI++  G L+L VTM  HP
Sbjct: 530 GSPTCFANQELLEKLTKLSLSHGKKLLIPAGALWGANDIQKMADVGSLKGLTVTMIKHPT 589

Query: 470 GFRLEGPLAAAHSSGP-----RTVLYEGPVRGLCPLAPRNSNTMAAAALAAPSLGFDRVI 306
       F+L PL +      TVLYEG VRGLCPLAP N NTMA  ALAA +LGFD V
Sbjct: 590 SFKLGSPLFEINEKAKLEETNETVLYEGSVRGLCPLAPNNVNTMAGGALAASNLGFDEVK 649

Query: 305 GVLVADLSLTDMHVVDVELTGPPGPTGRSFAVHTHRENPAQPGAVT 168
       L++D +TD HVV+V+ + G G   F V T R NPA+PGAVT
Sbjct: 650 AKLISDPKMTDWHVVEVRVEGDDG-----FEVITRRNNPAKPGAVT 690
```

## RISKMARKER3

RISKMARKER3 is a 1019 nucleotide sequence encoding superoxide dismutase copper chaperone [AF255305]:

```
1    ggtctctgga ccctaccggt tgtgtggccc aagcgggtga ctgcagccag gatggcttcg
61   aagtcggggg acggtggaac tatgtgtgcg ttggagttta cagtacagat gagttgtcag
121  agctgcgtgg acgctgtgca caagaccctg aaaggggcgg cgggtgtcca gaatgtggaa
181  gttcagttgg agaaccagat ggtgttggtg cagaccactt tgcccagcca ggaggtgcaa
241  gcgctcctgg aaagcacagg gaggcaggct gtactcaagg gcatgggcag cagccaacta
301  aagaatctgg gagcagcagt ggccattatg gagggcagtg gcaccgtaca gggggtggtc
361  cgcttcctac agctgtcctc tgagctctgc ctgattgagg gaaccatcga cggcctggag
421  cctgggctgc atgggcttca tgtccatcag tatggggacc ttaccaagga ctgcagcagc
```

```
481   tgtggggacc attttaaccc tgatggagca tctcatgggg gtcctcagga
cactgatcgg 541    caccggggag atctgggcaa tgttcacgct gaagctagtg
gccgagctac cttccggata 601     gaggataaac agctgaaggt gtgggatgtg
attggccgca gtctggttgt tgatgaggga 661      gaagatgacc tgggccgggg
aggccatccc ttatccaagg tcacagggaa ttctgggaag 721       aggttggcct
gtggcatcat tgcacgctct gctggccttt tccagaatcc caagcagatc 781  tgctcctgtg
atgggctcac tatctgggag gagcgaggcc ggcccattgc tggccaaggc 841  cgaaaggact
cagcccaacc ccctgctcac ctctgaacag agcctcctgt caggttattc 901  agtcctccta
gctgaacatc ttcctgcaga gggagcctca agcccttgct tgtataggcc 961  taaagggcag
ataggcattg ttgtatcctg agcaaattaa attgttactc tcatatggc
```

**RISKMARKER4**

RISKMARKER4 is a 878 nucleotide sequence encoding alpha-2 microglubulin [U31287]:

```
1     ggcacgagca gagagattgt cccaacagag aggcaattct attccctacc
aacatgaagc
61    tgttgctgct gctgctgtgt ctgggcctga cactggtctg tggccatgca
gaagaagcta 121    gttccacaag agggaacctc gatgtggcta agctcaatgg
ggattggttt tctattgtcg 181     tggcctctaa caaaagagaa aagatagaag
agaatggcag catgagagtt tttatgcagc 241      acatcgatgt cttggagaat
tccttaggct tcaagttccg tattaaggaa aatggagagt 301       gcagggaact
atatttggtt gcctacaaaa cgccagagga tggcgaatat tttgttgagt 361  atgacggagg
gaatacattt actatactta agacagacta tgacagatat gtcatgtttc 421  atctcattaa
tttcaagaac ggggaaacct tccagctgat ggtgctctac ggcagaacaa 481  aggatctgag
ttcagacatc aaggaaaagt ttgcaaaact atgtgaggcg catggaatca 541  ctagggacaa
tatcattgat ctaaccaaga ctgatcgctg tctccaggcc cgaggatgaa 601  gaaaggcctg
agcctccagt gctgagtgga gacttctcac caggactcta gcatcaccat 661  ttcctgtcca
tggagcatcc tgagacaaat tctgcgatct gatttccatc ctctgtcaca 721  gaaaagtgca
atcctggtct ctccagcatc ttccctaggt tacccaggac aacacatcga 781  gaattaaaag
ctttcttaaa tttctcttgg ccccacccat gatcattccg cacaaatatc 841  ttgctcttgc
agttcaataa atgattaccc ttgcactt
```

**RISKMARKER5**

RISKMARKER5 is a 2443 bp rat mRNA for Mx3 protein [X52713]. The nucleic acid was initially identified in a cloned fragment (having 100% sequence identity to the rat mRNA) having the following sequence:

```
   1 CCATGGATGAAATCTTCCAGCATCTGAATGCCTACCGCCAGGAGGCTCACAACTGCATCTCCAGCCACATTCCATTGATC

  81 ATCCAGTATTTCATCTTGAAGATGTTTGCTGAGAAGCTGCAGAAGGGCATGCTCCAGCTCCTGCAGGACAAGGATTCCTG

 161 CAGCTGGCTCCTGAAGGAAAAGAGTGACACCAGTGAGAAGAGGAGATTCCTGAAGGAGCGGTTGGCAAGGCTGGCCCAAG

 241 CTCAGCGCAGGCTAGC  (SEQ ID NO: 5)
```

Blast-N Results:

**>gb:GENBANK-ID:RNMX3|acc:X52713 Rat mRNA for Mx3 protein - Rattus norvegicus, 2443 bp.**

Top Previous Match Next Match

Length = 2443

```
Plus Strand HSPs:

Score = 1280 (192.1 bits), Expect = 9.5e-52, P = 9.5e-52
Identities = 256/256 (100%), Positives = 256/256 (100%), Strand = Plus / Plus

Query:     1 CCATGGATGAAATCTTCCAGCATCTGAATGCCTACCGCCAGGAGGCTCACAACTGCATCT 60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1710 CCATGGATGAAATCTTCCAGCATCTGAATGCCTACCGCCAGGAGGCTCACAACTGCATCT 1769

Query:    61 CCAGCCACATTCCATTGATCATCCAGTATTTCATCTTGAAGATGTTTGCTGAGAAGCTGC 120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1770 CCAGCCACATTCCATTGATCATCCAGTATTTCATCTTGAAGATGTTTGCTGAGAAGCTGC 1829

Query:   121 AGAAGGGCATGCTCCAGCTCCTGCAGGACAAGGATTCCTGCAGCTGGCTCCTGAAGGAAA 180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1830 AGAAGGGCATGCTCCAGCTCCTGCAGGACAAGGATTCCTGCAGCTGGCTCCTGAAGGAAA 1889

Query:   181 AGAGTGACACCAGTGAGAAGAGGAGATTCCTGAAGGAGCGGTTGGCAAGGCTGGCCCAAG 240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:  1890 AGAGTGACACCAGTGAGAAGAGGAGATTCCTGAAGGAGCGGTTGGCAAGGCTGGCCCAAG 1949

Query:   241 CTCAGCGCAGGCTAGC 256
             ||||||||||||||||
Sbjct:  1950 CTCAGCGCAGGCTAGC 1965
```

Blast-X Results:

**>ptnr:SWISSPROT-ACC:P18590 INTERFERON-INDUCED GTP-BINDING PROTEIN MX3 - Rattus norvegicus (Rat), 659 aa.**

```
Top  Previous Match  Next Match
            Length = 659

  Plus Strand HSPs:

 Score = 429 (151.0 bits), Expect = 5.3e-39, P = 5.3e-39
 Identities = 84/84 (100%), Positives = 84/84 (100%), Frame = +3

Query:     3 MDEIFQHLNAYRQEAHNCISSHIPLIIQYFILKMFAEKLQKGMLQLLQDKDSCSWLLKEK 182
             MDEIFQHLNAYRQEAHNCISSHIPLIIQYFILKMFAEKLQKGMLQLLQDKDSCSWLLKEK
Sbjct:   571 MDEIFQHLNAYRQEAHNCISSHIPLIIQYFILKMFAEKLQKGMLQLLQDKDSCSWLLKEK 630

Query:   183 SDTSEKRRFLKERLARLAQAQRRL 254
             SDTSEKRRFLKERLARLAQAQRRL
Sbjct:   631 SDTSEKRRFLKERLARLAQAQRRL 654
```

## RISKMARKER6

RISKMARKER6 is 369 bp novel gene fragment, which has 98% amino acid identity (90% nucleic acid sequence identity) to Human ERj3 protein [AJ250137]. The nucleic acid sequence was initially identified in a cloned fragment having the following sequence:

```
  1 TCTAGAAAGTCACCTTGGAAGAAGTGTACGCAGGGAACTTTGTGGAAGTAGTTAGAAACAAGCCCGTAGCCAGGCAGGCT

 81 CCTGGCAAACGGAAATGCAACTGTCGGCAGGAGATGCGAACCACACAGCTGGGACCAGGGCGCTTCCAAATGACCCAGGA

161 AGTGGTTTGTGACGAGTGCCCTAATGTCAAACTAGTGAATGAAGAACGAACACTAGAAGTGGAAATAGAGCCTGGGGTGA

241 GAGATGGCATGGAGTACCCCTTTATTGGAGAAGGTGAGCCTCATGTGGATGGGGAACCCGGAGACTTACGGTTCCGAATC

321 AAAGTTGTCAAGCACCGGATATTTGAGAGGAGAGGGGATGACCTGTACA  (SEQ ID NO: 6)
```

Blast-N Results:

**>gb:GENBANK-ID:HSA250137|acc:AJ250137 Homo sapiens mRNA for ERj3 protein (ERj3 gene) - Homo sapiens, 1159 bp.**

```
Top  Previous Match  Next Match
            Length = 1159

  Plus Strand HSPs:

 Score = 1524 (228.7 bits), Expect = 5.6e-63, P = 5.6e-63
```

```
Identities = 334/369 (90%), Positives = 334/369 (90%), Strand = Plus / Plus

Query:     1 TCTAGAAAGTCACCTTGGAAGAAGTGTACGCAGGGAACTTTGTGGAAGTAGTTAGAAACA 60
             TCTAGAA GTCAC TTGGAAGAAGT TA GCAGG AA TTTGTGGAAGTAGTTAGAAACA
Sbjct:   431 TCTAGAA-GTCACTTTGGAAGAAGTATATGCAGGAAATTTTGTGGAAGTAGTTAGAAACA 489

Query:    61 AGCCCGTAGCCAGGCAGGCTCCTGGCAAACGGAAATGCAACTGTCGGCAGGAGATGCGAA 120
             A CC GT GC AGGCAGGCTCCTGGCAAACGGAA TGCAA TGTCGGCA GAGATGCG A
Sbjct:   490 AACCTGTGGCAAGGCAGGCTCCTGGCAAACGGAAGTGCAATTGTCGGCAAGAGATGCGGA 549

Query:   121 CCACACAGCTGGGACCAGGGCGCTTCCAAATGACCCAGGAAGTGGTTTGTGACGAGTGCC 180
             CCAC CAGCTGGG CC GGGCGCTTCCAAATGACCCAGGA GTGGT TG GACGA TGCC
Sbjct:   550 CCACCCAGCTGGGCCCTGGGCGCTTCCAAATGACCCAGGAGGTGGTCTGCGACGAATGCC 609

Query:   181 CTAATGTCAAACTAGTGAATGAAGAACGAACACTAGAAGTGGAAATAGAGCCTGGGGTGA 240
             CTAATGTCAAACTAGTGAATGAAGAACGAAC CT GAAGT GAAATAGAGCCTGGGGTGA
Sbjct:   610 CTAATGTCAAACTAGTGAATGAAGAACGAACGCTGGAAGTAGAAATAGAGCCTGGGGTGA 669

Query:   241 GAGATGGCATGGAGTACCCCTTTATTGGAGAAGGTGAGCCTCATGTGGATGGGGAACCCG 300
             GAGA GGCATGGAGTACCCCTTTATTGGAGAAGGTGAGCCTCA GTGGATGGGGA CC G
Sbjct:   670 GAGACGGCATGGAGTACCCCTTTATTGGAGAAGGTGAGCCTCACGTGGATGGGGAGCCTG 729

Query:   301 GAGACTTACGGTTCCGAATCAAAGTTGTCAAGCACCGGATATTTGAGAGGAGAGGGGATG 360
             GAGA TTACGGTTCCGAATCAAAGTTGTCAAGCACC  ATATTTGA AGGAGAGG GATG
Sbjct:   730 GAGATTTACGGTTCCGAATCAAAGTTGTCAAGCACCCAATATTTGAAAGGAGAGGAGATG 789

Query:   361 ACCTGTACA 369
             A  TGTACA
Sbjct:   790 ATTTGTACA 798
```

Blast-X Results:

**>ptnr:SPTREMBL-ACC:Q9UBS4 ERJ3 PROTEIN PRECURSOR - Homo sapiens (Human), 358 aa.**

Top Previous Match Next Match

Length = 358

Plus Strand HSPs:

```
Score = 637 (224.2 bits), Expect = 2.1e-61, P = 2.1e-61
Identities = 119/121 (98%), Positives = 120/121 (99%), Frame = +3

Query:     6 KVTLEEVYAGNFVEVVRNKPVARQAPGKRKCNCRQEMRTTQLGPGRFQMTQEVVCDECPN 185
             +VTLEEVYAGNFVEVVRNKPVARQAPGKRKCNCRQEMRTTQLGPGRFQMTQEVVCDECPN
Sbjct:   139 EVTLEEVYAGNFVEVVRNKPVARQAPGKRKCNCRQEMRTTQLGPGRFQMTQEVVCDECPN 198

Query:   186 VKLVNEERTLEVEIEPGVRDGMEYPFIGEGEPHVDGEPGDLRFRIKVVKHRIFERRGDDL 365
             VKLVNEERTLEVEIEPGVRDGMEYPFIGEGEPHVDGEPGDLRFRIKVVKH IFERRGDDL
Sbjct:   199 VKLVNEERTLEVEIEPGVRDGMEYPFIGEGEPHVDGEPGDLRFRIKVVKHPIFERRGDDL 258

Query:   366 Y 368
             Y
Sbjct:   259 Y 259
```

## RISKMARKER7

RISKMARKER7 is a 594 bp novel gene fragment, which has 65% sequence identity to *Mus musculus* hexokinase II [AJ238540], probable 3' UTR. The nucleic acid sequence was initially identified in a cloned fragment having the following sequence:

```
  1 GGGCCCCACTAAAACATACACAAAAGAATAAAAATGTTCATTTTAAACTTAAACTGCTTCCTGGTTTTACAAGGCATAAA

 81 TATATAGCATCTCCAACAGCTACCTGTAGATTCTGTTAGTGCAAAACCTTAGAAACCCTCCTGGAGCTCAAAGGCATCCG

161 GACTAGT  (SEQ ID NO: 7)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
   1 TTTTTTTTTTTTTTTTAAAAAAGATTATAAAATTGAATTTATTGAGTTTCACACAAGATGCACTTATAAAATTAGTACT

  81 GAATGCCATTATGACAGAAGTGAGCATCATCCACACTCCCAAGAGCATCTGCAAAGGAAATCAATCTTCAGAGAATAGCA

 161 CAGAAACAGAAAATCCAAGCGAACAAAAAGATACATCTAGGCCGTGTTCTTGTTCTGACCAGGGCCGCATTTGGCAAAGC

 241 TTTCTCTGCACCTCCCCTGGTTGCCAAGGATACTTTCTTTTGTTAAAAAAAAAAGTTTAGAAGTGGGGCCCCACTAAAAC

 321 ATACACAAAAGAATAAAAATGTTCATTTTAAACTTAAACTGCTTCCTGGTTTTACAAGGCATAAATATATAGCATCTCCA

 401 ACAGCTACCTGTAGATTCTGTTAGTGCAAAACCTTAGAAACCCTCCTGGAGCTCAAAGGCATCCGGACTAGTTTTGTACT

 481 TAAACAGGATACGGGTAAACCACTTAAAATTTGCCATCTCTGCCCAAAGTGTTTGCATGAGAACTGAGTTTCAGAAGACA

 561 GCATAGGAAAGAGTCAGAAACGGTCAACTTTTTT  (SEQ ID NO: 8)
```

Blast-N Results:

**>gb:GENBANK-ID:MMU238540|acc:AJ238540 Mus musculus mRNA for hexokinase II - Mus musculus, 5474 bp.**

Top Previous Match Next Match

Length = 5474

Minus Strand HSPs:

```
 Score = 251 (37.7 bits), Expect = 0.045, P = 0.044
 Identities = 121/184 (65%), Positives = 121/184 (65%), Strand = Minus / Plus

Query:   184 GTTCGCTTGGATTTT-CTG-TTTCTGTGCTATTCTCTGAAGATT-GATTTCCTTTGCAGA 128
             G TC CT  G T  T CTG TTT TGTG T TTC  TGAA  TT GA T C T T CA A
Sbjct:  5287 GCTCTCTCTGCTAATGCTGCTTTGTGTGATCTTCAGTGAACCTTTGACT-CATCT-CATA 5344

Query:   127 TGCTCTTGGGAGTGTGGATGATGCTCACTTCTGTCATAATGG-CATTC-AGTACTAATTT 70
             T C CT GG A T  G  T  TG  C  TT TGTCAT ATG  CA T  AG ACTA TT
Sbjct:  5345 TCC-CTGGGCACTCGGTCTAGTGAGCGTTT-TGTCATCATGTACAGTAGAGAACTAGTTG 5402

Query:    69 TATAAGTGCATCTTGTGTGAAACTCA-ATAAATTCAATTTTATAATCTTTTTTAAAAAAA 11
              AT A   CAT T  TGT AA CT   AT AAT  AATTTTA   T TTTTTT AAAAAA
Sbjct:  5403 AATTAAC-CATGTGATGTTAA-CTATTATTAATA-AATTTTAACTTTTTTTTTCAAAAAA 5459

Query:    10 AAAAAAAAAA 1
             AAAAAAAAAA
Sbjct:  5460 AAAAAAAAAA 5469
```

```
 Score = 250 (37.5 bits), Expect = 0.051, P = 0.049
 Identities = 122/184 (66%), Positives = 122/184 (66%), Strand = Minus / Plus

Query:   184 GTTCGCTTGGATTTT-CTG-TTTCTGTGCTATTCTCTGAAGATT-GATTTCCTTTGCAGA 128
             G TC CT  G T  T CTG TTT TGTG T TTC  TGAA  TT GA T C T T CA A
Sbjct:  5287 GCTCTCTCTGCTAATGCTGCTTTGTGTGATCTTCAGTGAACCTTTGACT-CATCT-CATA 5344

Query:   127 TGCTCTTGGGAGTGTGGATGATGCTCACTTCTGTCATAATGG-CATTC-AGTACTAATTT 70
             T C CT GG A T  G  T  TG  C  TT TGTCAT ATG  CA T  AG ACTA TT
Sbjct:  5345 TCC-CTGGGCACTCGGTCTAGTGAGCGTTT-TGTCATCATGTACAGTAGAGAACTAGTTG 5402

Query:    69 TATAAGTGCATCTTGTGTGAAACTCA-ATAAATTCAATTTTATAATCTTTTTT-AAAAAA 12
              AT A   CAT T  TGT AA CT   AT AAT  AATTTTA   T TTTTTT AAAAAA
Sbjct:  5403 AATTAAC-CATGTGATGTTAA-CTATTATTAATA-AATTTTAACTTTTTTTTTCAAAAAA 5459

Query:    11 AAAAAAAAAAA 1
             AAAAAAAAAAA
Sbjct:  5460 AAAAAAAAAAA 5470
```

Blast-X Results:

**>ptnr:SPTREMBL-ACC:Q9VIA2 MST84DB PROTEIN - Drosophila melanogaster (Fruit fly), 70 aa.**

Top Previous Match Next Match

Length = 70

Plus Strand HSPs:

```
 Score = 66 (23.2 bits), Expect = 2.2, P = 0.88
 Identities = 15/48 (31%), Positives = 25/48 (52%), Frame = +3
```

```
Query:    66 YKISTECHYDRSEHHPHSQEHLQRKS------IFRE*HRNRKSKRTKR 191
             YK+ ++ H  R +H P S++   RK       I ++  RNRK  R ++
Sbjct:     3 YKVHSKVHKARMDHSPRSKDRKDRKGRKAHSKIHKDYSRNRKDHRVRK 50
```

## RISKMARKER8

RISKMARKER8 is a 797 bp novel gene fragment, which has 94% amino acid identity (79% nucleic acid sequence identity) to human GT335 mRNA (ES1 Protein Homolog) [U53003]. The nucleic acid was initially identified in a cloned fragment having the following sequence:

```
   1 CCTAGGACTGCACAACGTGAGTCCTTGAACCAGGCTCTGGAAAAGGTGCCCAGACCACCCAATGGGGACACACAGTGAGG
  81 CCAGCCCCCAGTGAAATTCCTGCTGCTACCTGGGGCCCTTGGTGAGACTCTGGCTTCCGGCTGGTAGAAGCCAAGGTTGG
 161 ACGCATAGTTGCAAAGCTCCTCCTTCAGGCACAAAGTGTCTATGCTTCTAATAGAACAGCAGCTCCCGTGTCCTGGCTGA
 241 CCGGAGCACACAGGCTGAGCGTGCCACAGCGACGACGGAGGCCAAGCGTGGTGCTGGTGGTGTTACTTTCCCGTGAGTTC
 321 CAGCACCTTCTTCACCATGG  (SEQ ID NO: 9)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
   1 TTTTTTTTTTTTTTTTTTTTTTGAGTTTCCACTGTGGAAAAGAGTTTATTGTATGGCTGCAGGGATCTACTACAGAATCC
  81 CCCTGGCTGCAGTTAGCTGTGCTTACTCTGGACATATCTCCGAAGACTTGGAGCCTAAATGGTTTTCTTCTTTTAGAGCT
 161 TTAGTACCCGATCCATCAGACCTAGGACTGCACAACGTGAGTCCTTGAACCAGGCTCTGGAAAAGGTGCCCAGACCACCC
 241 AATGGGGACACACAGTGAGGCCAGCCCCCAGTGAAATTCCTGCTGCTACCTGGGGCCCTTGGTGAGACTCTGGCTTCCGG
 321 CTGGTAGAAGCCAAGGTTGGACGCATAGTTGCAAAGCTCCTCCTTCAGGCACAAAGTGTCTATGCTTCTAATAGAACAGC
 401 AGCTCCCGTGTCCTGGCTGACCGGAGCACACAGGCTGAGCGTGCCACAGCGACGACGGAGGCCAAGCGTGGTGCTGGTGG
 481 TGTTACTTTCCCGTGAGTTCCAGCACCTTCTTCACCATGGCCCCAATCCCGTCGTGGATGTGGTGGAGTTCGGTCTCACA
 561 CATGAAGGCCGGGGTGGTGACCACCTTGTTTTTCTGGTCGACGTGAGCTTCGGTCACACCCTTCACACAGTGCTTGGCAC
 641 CCAGGGCTTTGACGGCTTCCGCGGTTCCAGCATATGGCCACTTGCCCCCCTCCTCTTGCTCATGGCCCACGGTGACCTCC
 721 ACACCTTTGATCACTTTGGCTGCGAGGACAGGAGCGATGCAGCATAGGCCAATGGGCTTCTTGGCTCCGTGGAATTC
     (SEQ ID NO: 10)
```

Blast-N Results:

**>gb:GENBANK-ID:HSU53003|acc:U53003 Human GT335 mRNA, complete cds - Homo sapiens, 1652 bp.**

Top Previous Match Next Match
Length = 1652

Minus Strand HSPs:

```
 Score = 1141 (171.2 bits), Expect = 7.9e-46, P = 7.9e-46
 Identities = 307/385 (79%), Positives = 307/385 (79%), Strand = Minus / Plus

Query:   797 GAATTCCACGGAGCCAAGAAGCCCATTGGCCTATGCTGCATCGCTCCTGTCCTCGCAGCC 738
             GA TTCCAC   GCC  GAAGCCCAT GGC T TGCTGCAT GC CCTGTCCTCGC GCC
Sbjct:   577 GAGTTCCACCAGGCCGGGAAGCCCATCGGCTTGTGCTGCATTGCACCTGTCCTCGCGGCC 636
```

```
Query:   737 AAAGTGATCAAAGGTGTGGAGGTCACCGTGGGCCATGAGCAAGAGGAGGGGGGCAAGTGG 678
             AA GTG TCA AGG GT GAGGT AC GTGGGCCA GAGCA GAGGA GG GGCAAGTGG
Sbjct:   637 AAGGTGCTCAGAGGCGTCGAGGTGACTGTGGGCCACGAGCAGGAGGAAGGTGGCAAGTGG 696

Query:   677 CCATATGCTGGAACCGCGGAAGCCGTCAAAGCCCTGGGTGCCAAGCACTGTGTGAAGGGT 618
             CC TATGC GG ACCGC GA GCC TCAA GCCCTGGGTGCCAAGCACTG GTGAAGG
Sbjct:   697 CCTTATGCCGGGACCGCAGAGGCCATCAAGGCCCTGGGTGCCAAGCACTGCGTGAAGGAA 756

Query:   617 GTGACCGAAGCTCACGTCGACCAGAAAAACAAGGTGGTCACCACCCCGGCCTTCATGTGT 558
             GTG  CGAAGCTCACGT GACCAGAAAAACAAGGTGGTCAC ACCCC GCCTTCATGTG
Sbjct:   757 GTGGTCGAAGCTCACGTGGACCAGAAAAACAAGGTGGTCACGACCCCAGCCTTCATGTGC 816

Query:   557 GAGACCGAACTCCACCACATCCACGACGGGATTGGGGCCATGGTGAAGAAGGTGCTGGAA 498
             GAGAC G ACTCCAC ACATCCA GA GGGAT GG GCCATGGTGA GAAGGTGCTGGAA
Sbjct:   817 GAGACGGCACTCCACTACATCCATGATGGGATCGGAGCCATGGTGAGGAAGGTGCTGGAA 876

Query:   497 CTCACGGGAAAGTAACAC-CACC-A-GCACCAC-GCTTGGCCTCCGT-CGTCGCTGTGGC 443
             CTCAC GGAAAGT AC C CA   A G   C C GCT GGC  C G  C T GC  T  C
Sbjct:   877 CTCACTGGAAAGTGACGCGCATGGACGGGGCCCAGCTAGGCGCCAGGACTTGGCC-T--C 933

Query:   442 ACGCTCAGCCTGTGT-GCTC-CGGTCAGC 416
             AC CTC G CTG G  GCT  CGG C GC
Sbjct:   934 ACCCTCTGGCTGAGGAGCTGTCGG-CTGC 961
```

Blast-X Results:

**>ptnr:SWISSNEW-ACC:P30042 ES1 PROTEIN HOMOLOG, MITOCHONDRIAL PRECURSOR (PROTEIN KNP-I) (GT335 PROTEIN) - Homo sapiens (Human), 268 aa.**

Top Previous Match Next Match
Length = 268

Minus Strand HSPs:

Score = 505 (177.8 bits), Expect = 2.0e-47, P = 2.0e-47
Identities = 94/104 (90%), Positives = 99/104 (95%), Frame = -1

```
Query:   797 EFHGAKKPIGLCCIAPVLAAKVIKGVEVTVGHEQEEGGKWPYAGTAEAVKALGAKHCVKG 618
             EFH A KPIGLCCIAPVLAAKV++GVEVTVGHEQEEGGKWPYAGTAEA+KALGAKHCVK
Sbjct:   165 EFHQAGKPIGLCCIAPVLAAKVLRGVEVTVGHEQEEGGKWPYAGTAEAIKALGAKHCVKE 224

Query:   617 VTEAHVDQKNKVVTTPAFMCETELHHIHDGIGAMVKKVLELTGK 486
             V EAHVDQKNKVVTTPAFMCET LH+IHDGIGAMV+KVLELTGK
Sbjct:   225 VVEAHVDQKNKVVTTPAFMCETALHYIHDGIGAMVRKVLELTGK 268
```

Principle components analysis was used to generate three eigenvectors used to transform the original expression level data matrix, as shown in Table 4 below. Eigenvector 1 values represent NSAIDs with low risk of hepatoxicity, Eigenvector 2 values represent NSAIDs with very low risk of hepatoxicity, and Eigenvector 3 values represent NSAIDs with overdose risk of hepatoxicity.

Table 4: Transform Eigenvectors for Hepatoxicity Markers by Risk Classification

| Gene | Eigenvector 1 | Eigenvector 2 | Eigenvector3 |
|---|---|---|---|
| RISKMARKER1 | 26.9 | 6.7 | -0.9 |
| RISKMARKER2 | 23.3 | -1.4 | 1.5 |
| RISKMARKER3 | -26.0 | -1.5 | -2.3 |
| RISKMARKER4 | 12.6 | -2.2 | -6.4 |
| RISKMARKER5 | 18.0 | -1.3 | -3.1 |
| RISKMARKER6 | -13.8 | 4.71 | 19.3 |
| RISKMARKER7 | -29.7 | -7.5 | 1.3 |
| RISKMARKER8 | 19.3 | 1.2 | -2.6 |
| % of variation explained | 99.6 | 0.4 | 0.1 |

These eigenvectors may be used to transform the expression levels of RISKMARKERS 1-8 ("RISKMARKERS") in response to a given drug, in order to determine that drug's hepatotoxicity risk. For example, expression levels of RISKMARKERS correlating with Eigenvector 1 indicates that the test drug has a low risk of hepatotoxicity. Alternatively, a drug's RISKMARKERS expression profile can be generated simultaneously with the above-described training set (or an equivalent set) run in parallel with the test drug, and expression levels associated with the test drug directly compared to those of the training set.

A second training set based on type of injury (hepatocellular damage, cholestasis, elevated transaminase level) was also constructed, utilizing the compounds indicated in Table 5, below.

Table 5: Training Set of NSAIDs by Injury Type

| Control | Hepatocellular | Cholestasis | Elevated transmainases |
|---|---|---|---|
| Sterile water | Acetaminophen | Benoxaprofen | Zomepirac |
| 10% ethanol | Flurbiprofen | Nabumetone | Mefenamic acid |
| Canola oil | Ketoprofen | Sulindac | Tenoxicam |

This analysis produced ten fragments that significantly (p=8.7 x 10-18) discriminated among the drugs in the test set. The identities of these ten fragments (INJURYMARKER 1-10) that are included in the discriminatory set (with GenBank accession numbers) are shown below. Where appropriate, the cloned sequence from isolation is provided, and this sequence was then extended using either Genbank rat ESTs or from internally sequenced (Curagen Corporation) rat fragments. The fragments were used to extend the cloned sequence, and the extended contig sequence is provided as "consensus." Finally, the best BlastN and BlastX results are also provided. In some instances the cloned sequence is identical to a known rat gene, in those instances the name of the gene and the GenBank accession number is provided.

**INJURYMARKER1**

INJURYMARKER1 is a 1025 bp rat express sequence tag (EST) [AI169175]. The nucleic acid was initially identified in a cloned fragment having the following sequence:

```
1   CTGATTTCAAATTTTTATTAGAGAACACTTTCGGATTTCAAATTTTTATTACAGAACAAACATTTTCTGATTTCAAATTT
81  CTATTATAATTCTCCAGTAATCAAAGCAGTGGCGTTGGCATGAAGGCAGACAGAGGTCATGGAAGAGACCAGGCTCAGAA
161 ACAGCCCCACCATGCACAGCGGGATGTTTTCCCACCAAGGGCAACATGCAAAGCCAGGTATCCACATGGGTAGAGTAGAA
```

```
241 AGTCAGACCTTACATCTCACACACAAATGAACTCAAAATATACCAGAGAGCAAAGCTAAGAGCTAAAATCAAGTTTCCTA

321 GGGCAAGCTGTAGTAGGCTCCCTTGGGTGGGTTAATGCTTTTGTGGATGTGACTACCAAAAATTCAACCAGAGCCAACGA

401 CCCAACTATTAATGGGCAGTGGACCTAAAGAGATTTCTTCAAACGATATATAAAGAAGGCCACCAAGCATATAAAACATG

481 TGACATCAGTAGTCAGAGAGATGGGAAGCAGAAGCACTAGCAGATCTTAACACCTACTAGAACANCCACTAAAAAAGAGT

561 AAGACTCACAAGGACATGGGCACTTCTAATCTCTGTGCACTGCTGCCAGGACATACAATAGTGTGGTCACTATGGAGACT

641 ACGGCAGTGCCTACTAATAACAGCAGAGTTACCCTAAGACATACAATCTGCTGCGTGTATGCTAAGCAGGATCCGAGGGA

    TATTTGTATATACATGTTCACAGCATAGTCAGGAGCTCCAGGGTGGGAACAACTGAGGTACC
721
    (SEQ ID NO: 11)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
1   CTGATTTCAAATTTTTATTATAGAACACTTTCTGATTTCAAATTTTTATTACAGAACAAACATTTTCTGATTTCAAATTT

81  CTATTATAATTCTCCAGTAATCAAAGCAGTGGCGTTGGCATGAAGGCAGACAGAGGTCATGGAAGAGACCAGGCTCAGAA

161 ACAGCCCCACCATGCACAGCGGGATGTTTTCCCACCAAGGGCAACATGCAAAGCCAGGTATCCACATGGGTAGAGTAGAA

241 AGTCAGACCTTACATCTCACACACAAATGAACTCAAAATATACCAGAGAGCAAAGCTAAGAGCTAAAATCAAGTTTCCTA

321 GGGCAAGCTGTAGTAGGCTCCCTTGGGTGGGTTAATGCTTTTGTGGATGTGACTACCAAAAATTCAACCAGAGCCAACGA

401 CCCAACTATTAATGGGCAGTGGACCTAAAGAGATTTCTTCAAACGATATATAAAGAAGGCCACCAAGCATATAAAACATG

481 TGACATCAGTAGTCAGAGAGATGGGAAGCAGAAGCACTAGCAGATCTTAACACCTACTAGAACAGCCACTAAAAAAGAGT

561 AAGACTCACAAGGACATGGGCACTTCTAATCTCTGTGCACTGCTGCCAGGACATACAATAGTGTGGTCACTATGGAGACT

641 ACGGCAGTGCCTACTAATAACAGCAGAGTTACCCTAAGACATACAATCTGCTGCGTGTATGCTAAGCAGGATCCGAGGGA

721 TATTTGTATATACATGTTCACAGCATAGTCAGGAGCTCCAGGGTGGGAACAACTGAGGTACCCACGGCTGGATGAGTAGG

801 TAACAAGAAACATACAGCATACATACAACACACACTAAAGTCTAAAGTACTATTTGTCCTTACAAAGGAAACTCATACAT

881 GATACAAGCCTTCACGGCATTCTGCTACATGAACACGCACACACACACACACACACACACACACACGCACTGAGAATC

    TATGTATACCAGGCACTTAGGGTACTCAAATTCAGAAACAGGACAGAGAATGGTGATTGCCATGG
961
    (SEQ ID NO: 12)
```

Blast-N Results:

**>gb:GENBANK-ID:AI169175|acc:AI169175 EST215009 Normalized rat kidney, Bento Soares Rattus sp. CDNA clone RKIBO44 3' end, mRNA sequence - Rattus sp., 670 bp (RNA).**

Top Previous Match Next Match
Length = 670

Plus Strand HSPs:

Score = 3305 (495.9 bits), Expect = 4.3e-143, P = 4.3e-143
Identities = 661/661 (100%), Positives = 661/661 (100%), Strand = Plus / Plus

```
Query:     4 ATTTCAAATTTTTATTATAGAACACTTTCTGATTTCAAATTTTTATTACAGAACAAACAT 63
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 ATTTCAAATTTTTATTATAGAACACTTTCTGATTTCAAATTTTTATTACAGAACAAACAT 60

Query:    64 TTTCTGATTTCAAATTTCTATTATAATTCTCCAGTAATCAAAGCAGTGGCGTTGGCATGA 123
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 TTTCTGATTTCAAATTTCTATTATAATTCTCCAGTAATCAAAGCAGTGGCGTTGGCATGA 120

Query:   124 AGGCAGACAGAGGTCATGGAAGAGACCAGGCTCAGAAACAGCCCCACCATGCACAGCGGG 183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 AGGCAGACAGAGGTCATGGAAGAGACCAGGCTCAGAAACAGCCCCACCATGCACAGCGGG 180
```

```
Query:   184 ATGTTTTCCCACCAAGGGCAACATGCAAAGCCAGGTATCCACATGGGTAGAGTAGAAAGT 243
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 ATGTTTTCCCACCAAGGGCAACATGCAAAGCCAGGTATCCACATGGGTAGAGTAGAAAGT 240

Query:   244 CAGACCTTACATCTCACACACAAATGAACTCAAAATATACCAGAGAGCAAAGCTAAGAGC 303
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   241 CAGACCTTACATCTCACACACAAATGAACTCAAAATATACCAGAGAGCAAAGCTAAGAGC 300

Query:   304 TAAAATCAAGTTTCCTAGGGCAAGCTGTAGTAGGCTCCCTTGGGTGGGTTAATGCTTTTG 363
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   301 TAAAATCAAGTTTCCTAGGGCAAGCTGTAGTAGGCTCCCTTGGGTGGGTTAATGCTTTTG 360

Query:   364 TGGATGTGACTACCAAAAATTCAACCAGAGCCAACGACCCAACTATTAATGGGCAGTGGA 423
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   361 TGGATGTGACTACCAAAAATTCAACCAGAGCCAACGACCCAACTATTAATGGGCAGTGGA 420

Query:   424 CCTAAAGAGATTTCTTCAAACGATATATAAAGAAGGCCACCAAGCATATAAAACATGTGA 483
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   421 CCTAAAGAGATTTCTTCAAACGATATATAAAGAAGGCCACCAAGCATATAAAACATGTGA 480

Query:   484 CATCAGTAGTCAGAGAGATGGGAAGCAGAAGCACTAGCAGATCTTAACACCTACTAGAAC 543
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   481 CATCAGTAGTCAGAGAGATGGGAAGCAGAAGCACTAGCAGATCTTAACACCTACTAGAAC 540

Query:   544 AGCCACTAAAAAAGAGTAAGACTCACAAGGACATGGGCACTTCTAATCTCTGTGCACTGC 603
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   541 AGCCACTAAAAAAGAGTAAGACTCACAAGGACATGGGCACTTCTAATCTCTGTGCACTGC 600

Query:   604 TGCCAGGACATACAATAGTGTGGTCACTATGGAGACTACGGCAGTGCCTACTAATAACAG 663
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   601 TGCCAGGACATACAATAGTGTGGTCACTATGGAGACTACGGCAGTGCCTACTAATAACAG 660

Query:   664 C 664
             |
Sbjct:   661 C 661
```

## INJURYMARKER2

INJURYMARKER2 is a 893 nucleotide sequence encoding phosphotidylethanolamine N-methyltransferase [L14441]:

```
1   tccccgctga gttcatcacc agggacaggt gacctgagct gcccctggag cccagctccc
61  atttccttct ggttctggcc gatctcttcg ttatgagctg gctgctgggt tacgtggacc
121 ccacagagcc cagctttgtg gcggctgtgc tcaccattgt gttcaatcca ctcttctgga
181 atgtggtagc aaggtgggag cagagaactc gcaagctgag cagagccttc gggtcccctt
241 acctagcctg ctattccctg ggcagcatca tcctgcttct gaacatcctc cgctcccact
301 gcttcacaca ggccatgatg agccagccca agatggaggg cctggatagc cacaccatct
361 acttcctggg ccttgcactc ctgggctggg gactcgtgtt tgtgctctcc agcttctatg
421 cactggggtt cactgggacc tttctaggtg actactttgg gatcctcaag gagtccagag
481 tgaccacatt tcccttcagc gtgctggaca accccatgta ctggggaagt acagccaact
541 acctaggctg ggcacttatg cacgccagcc ctacaggcct gctgttgacg gtgctggtgg
601 cactcgtcta cgtggttgct ctcctgtttg aagagccctt cactgcggag atctaccggc
661 ggaaagccac caggttgcac aaaaggagct gacagggcca tgagggacct ttggaaagcc
721 ggattgcctc ccggctgacc caagcaacaa cccttctcgg ggagagcagc gctggccatt
781 gtacctgtgc cttggaaacc agtcatgggg gtgctcaggc attatgtcat gtgactgctg
841 agacccccat ccccaccaat ccctgacaca ctaataaagg ctttgtgacc tcc
```

## INJURYMARKER3

INJURYMARKER3 is a 1131 nucleotide hexokinase-encoding sequence [M86235]:

```
1    agcaggaatc ccctccgctt gcgggtagga agcttgggga gcagcctcat
60   ggaagagaag
```

```
61    cagatcctgt gcgtggggct ggtggtgctg gacatcatca atgtggtgga
caaataccca
121   gaggaagaca cggatcgcag gtgcctatcc cagagatggc agcgtggagg
caacgcgtcc
181   aactcctgca ctgtgctttc cttgctcgga gcccgctgtg ccttcatggg
ctcgctggcc
241   catggccatg ttgccgactt cctggtggcc gacttcaggc ggaggggtgt
ggatgtgtct
301   caagtggcct ggcagagcca gggagatacc ccttgctcct gctgcatcgt
caacaactcc
361   aatggctccc gtaccattat tctctacgac acgaacctgc cagatgtgtc
tgctaaggac
421   tttgagaagg tcgatctgac ccggttcaag tggatccaca ttgagggccg
gaatgcatcg
481   gaacaggtaa agatgctaca gcggatagaa cagtacaatg ccacgcagcc
tctgcagcag
541   aaggtccggg tgtccgtgga gatagagaag ccccgagagg aactcttcca
gctgttcggc
601   tatggagagg tggtgtttgt cagcaaagat gtggccaagc acctggggtt
ccggtcagca
661   ggggaggccc tgaagggctt gtacagtcgt gtgaagaaag gggctacgct
catctgtgcc
721   tgggctgagg agggagccga tgccctgggc cccgacggcc agctgctcca
ctcagatgcc
781   ttcccaccac cccgagtagt agacactctc ggggctggag acaccttcaa
tgcctctgtc
841   atcttcagcc tctccaaggg aaacagcatg caggaggccc tgagattcgg
gtgccaggtg
901   gctggcaaga agtgtggctt gcaggggttt gatggcattg tgtgagagat
gagcggtggg
961   aggtagcagc tcgacacctc agaggctggc accactgcct gccattgcct
tcttcatttc
1021  atccagcctg gcgtctggct gcccagttcc ctgggccagt gtaggctgtg
gaacgggtct
1081  ttctgtctct tctctgcaga cacctggagc aaataaatct tcccctgagc c
```

## INJURYMARKER4

INJURYMARKER4 is a 1994 nucleotide sequence encoding mitochondrial HMG-CoA Synthase [M33648]:

```
1     atctctccca ggggctgtgg actgctggct ttctgttgat accttagaga
tgcagcggct
61    tttggctcca gcaaggcggg tcctgcaagt gaagagagtc atgcaggaat
cttcgctctc 121    acccgctcac ctgctccccg cagcccagca gaggttttct
acaatccctc ctgctcccct 181     ggccaaaact gatacatggc caaaagatgt
gggcatcctt gccctggagg tctactttcc 241      agcccaatat gtggaccaaa
ctgacctgga gaagttcaac aatgtggaag cagggaagta 301       cacagtgggc
ttgggccaga cccgtatggg cttctgttcg gtccaggagg acatcaactc 361  cttgtgcctc
acagtggtgc agaggctgat ggaacgcaca aagctgccat gggatgccgt 421  aggccgcctg
gaagtgggca cggaaaccat cattgacaag tccaaggctg tcaagacagt 481  gctcatggag
ctcttccagg attcaggcaa cactgacatc gagggcatag ataccaccaa 541  cgcctgctat
ggtggcactg cctccctctt caacgctgcc aactggatgg agtccagcta 601  ctgggatggt
cgctatgccc tggtggtctg tggtgatatc gcagtctacc caagtggtaa 661  cccccgcccc
acaggtggtg ccggggctgt ggcaatgctg attgggccca aggccccgct 721  agtcctggaa
caagggctga ggggaaccca catggagaac gcctatgact tctacaaacc 781  aaacttggcc
tcagagtatc cactggtgga tgggaagctg tctatccagt gctacctgcg 841  ggccttggac
```

cgatgctatg cagcttaccg caggaaaatc cagaatcagt ggaagcaagc 901 tggaaacaac
cagcctttca ccctcgatga cgtgcaatat atgatcttcc acacaccctt 961 ttgcaagatg
gtccagaaat ccctagctcg gctgatgttc aatgacttcc tgtcatctag 1021
cagtgacaag cagaacaact tatacaaggg tctagaggcc ttcaagggtc
taaagctgga 1081 agaaacctac accaacaagg atgttgacaa ggctctgctg
aaggcctccc tggacatgtt 1141 caacaagaaa accaaggcct ccctttacct
ctccacaaac aatgggaaca tgtacacctc 1201 gtccctctac gggtgcctgg
cctcacttct ctcccaccac tctgcccaag aattggccgg 1261 ctccaggatt
ggagccttct cctacggctc aggcttagca gcaagtttct tctcatttcg 1321
agtgtccaag gacgcttccc caggttcccc tctggagaag ctggtgtcta
gtgtgtcaga 1381 tctgcccaaa cgtctagact cccggagacg catgtcccct
gaggaattca cagaaataat 1441 gaatcagaga gagcaatttt accacaaggt
gaacttctct ccccctggtg acacaagcaa 1501 cctcttccca ggcacttggt
accttgaacg agtggatgag atgcaccgca gaaaatatgc 1561 ccggcgtccc
gtctaaggag accaatccat acaaccattc cccggggaaa gaatgtgagc 1621
agagccgtta cccaaacggc ttccacttaa aattccaccc acagcagtga
acggtgaata 1681 gacacagcga ccccatagga tctgctccgc ggtgaagggc
ctccctctgt ggatcctggg 1741 tgaccctccc tgaagcagtg agcaccacag
gttctgctgt ggaccagagc ccccctgtgg 1801 agagggagaa agaaagggga
gccgctgacc tgcagggata cagaccttcc ccacagcctg 1861 gcagccgccc
gtttgttgca gcttattatc agactgtggg ctatcatagt tcatgctcgt 1921
ttcttaaagt ttcccgagaa tttctaaaat tttgtatcta aacttttaat
atggcgatta 1981 aaaggagaga agga

## INJURYMARKER5

INJURYMARKER5 is a 1850 nucleotide sequence encoding cathepsin C [D90404], having the following nucleic acid sequence:

1 gaattccggt tctagttgtt gttttctctg ccatctgctc tccgggcgcc gtcaaccatg
61 ggtccgtgga cccactcctt gcgcgccgcc ctgctgctgg tgcttttggg agtctgcacc
121 gtgagctccg acactcctgc caactgcact taccctgacc tgctgggtac ctgggttttc
181 caggtgggcc ctagacatcc ccgaagtcac attaactgct cggtaatgga accaacagaa
241 gaaaaggtag tgatacacct gaagaagttg gatactgcct atgatgaagt gggcaattct
301 gggtatttca ccctcattta caaccaaggc tttgagattg tgttgaatga
ctacaagtgg 361 tttgcgtttt tcaagtatga agtcaaaggc agcagagcca
tcagttactg ccatgagacc 421 atgacagggt gggtccatga tgtcctgggc
cggaactggg cttgctttgt tggcaagaag 481 atggcaaatc actctgagaa
ggtttatgtg aatgtggcac accttggagg tctccaggaa 541 aaatattctg
aaaggctcta cagtcacaac cacaactttg tgaaggccat caattctgtt 601 cagaagtctt
ggactgcaac cacctatgaa gaatatgaga aactgagcat acgagatttg 661 ataaggagaa
gtggccacag cggaaggatc ctaaggccca aacctgcccc gataactgat 721 gaaatacagc
aacaaatttt aagtttgcca gaatcttggg actggagaaa cgtccgtggc 781 atcaattttg
ttagccctgt tcgaaaccaa gaatcttgtg gaagctgcta ctcatttgcc 841 tctctgggta
tgctagaagc aagaattcgt atattaacca acaattctca gaccccaatc 901 ctgagtcctc
aggaggttgt atcttgtagc ccgtatgccc aaggttgtga tggtggattc 961 ccatacctca
ttgcaggaaa gtatgcccaa gattttgggg tggtggaaga aaactgcttt 1021
ccctacacag ccacagatgc tccatgcaaa ccaaaggaaa actgcctccg
ttactattct 1081 tctgagtact actatgtggg tggtttctat ggtggctgca
atgaagccct gatgaagctt 1141 gagctggtca aacacggacc catggcagtt
gcctttgaag tccacgatga cttcctgcac 1201 taccacagtg ggatctacca
ccacactgga ctgagcgacc ctttcaaccc ctttgagctg 1261 accaatcatg
ctgttctgct tgtgggctat ggaaaagatc cagtcactgg gttagactac 1321
tggattgtca agaacagctg gggctctcaa tggggtgaga gtggctactt
ccggatccgc 1381 agaggaactg atgaatgtgc aattgagagt atagccatgg
cagccatacc gattcctaaa 1441 ttgtaggacc tagctcccag tgtcccatac
agctttttat tattcacagg gtgatttagt 1501 cacaggctgg agacttttac
aaagcaatat cagaagctta ccactaggta cccttaaaga 1561 attttgccct

taagtttaaa acaatccttg attttttttct tttaatatcc tccctatcaa 1621
tcaccgaact acttttcttt ttaaagtact tggttaagta atacttttct
gaggattggt 1681 tagatattgt caaatatttt tgctggtcac ctaaaatgca
gccagatgtt tcattgttaa 1741 aaatctatat aaaagtgcaa gctgcctttt
ttaaattaca taaatcccat gaatacatgg 1801 ccaaaatagt tatttttttaa
agactttaaa ataaatgatt aatcgatgct

## INJURYMARKER6

INJURYMARKER6 is a 993 nucleotide sequence encoding hydroxysteroid sulfotransferase [D14989]:

```
1    ggcaagggct ggaatactaa aagttattca tgatgtcaga ctatacttgg tttgaaggaa
61   tacctttttcc tgccttttgg ttttccaaag aaattctgga aaatagttgt aagaagtttg
121  tggtaaaaga agacgacttg atcatattga cttaccccaa gtcaggaacg aactggctga
181  tcgagattgt ctgcttgatt cagaccaagg gagatcccaa gtggatccaa tctatgccca
241  tctgggatcg ctcaccctgg atagagactg gttcaggata tgataaatta accaaaatgg
301  aaggaccacg actcatgacc tcccatcttc ccatgcatct tttctccaag tctctcttca
361  gttccaaggc caaggtgata tatctcatca gaaatcccag agatgttctt gtttctgctt
421  atttttttctg gagtaagatc gccctggaga agaaaccaga ctcgctggga acttacgttg
481  aatggttcct caaaggaaat gttgcatatg gatcatggtt tgagcacatc cgtggctggc
541  tgtctatgag agaatgggac aacttcttgg tactgtacta tgaagacatg aaaaaggata
601  caatgggatc cataaagaag atatgtgact tcctggggaa aaaattagag ccagatgagc
661  tgaatttggt cctcaagtat agttccttcc aagtcgtgaa agaaaacaac atgtccaatt
721  atagcctcat ggagaaggaa ctgattctta ctggttttac tttcatgaga aaaggcacaa
781  ctaatgactg gaagaatcac ttcacagtag cccaagctga agcctttgat aaagtgttcc
841  aggagaaaat ggccggtttc cctccaggga tgttcccatg ggaataaatt ttcaaaagtt
901  ttaaatattt tatgaacact gatgtttatg tttatgttgt tctatgatgt ctgaataact
961  gaatgtgatc attgaataaa tcctgttgtg gat
```

## INJURYMARKER7

INJURYMARKER7 is a 5001 nucleotide sequence encoding insulin-like growth factor binding protein [L22979]:

```
1    cacaaaccca gcgagcattg aacactgcac acggccatct gcccagagag ctgtgaccac
61   cacttccgct actatctact cagaaagtcg tgactactga gccactgctg cctgcccaga
121  ttctcatcca ccgcctgctg cgtctggttg cgatgccgga gttcctaact gttgtttctt
181  ggccgttcct gatcctcctg tccttccagg ttcgcgtagt cgctggagcc ccccagccat
241  ggcactgtgc tccctgcact gctgagaggc tggagctctg tccacccgtg cctgcttcgt
301  gccccgagat ttctcggcct gcgggctgtg gctgctgccc gacatgtgcc ttgccactgg
361  gtgctgcctg tggtgtggcc actgcggcct gcgctcaggg actcagctgc cgtgcgctgc
421  caggggagcc tcgacctctg catgccctca cccgtggcca gggagcctgt gtactagaac
481  ctgccgcacc cgccacgagc agcttgtccg gttctcagca tgaaggtact acagccctct
541  ctgcctcttg atctcttggc taggacacac gtgctttcta ggcacgtcag aggcctatcc
601  ggaacctata gcagatagga caaaggctct ccatgcccac tttgagcttt cagcctcaaa
661  taaggccctc agttaggtcg tggcggcttg ggaaacacca gaggtgtcaa tccagtagca
721  gagtggagaa gttgggaaga atgttccaag ctcccagtgc agagtggaga gttgggaaga
781  atgttcacag actaggtagt actgatcctg cttggtcttt cagtggggag ggagctatgg
841  ggctgccagg tgggtggggt gctggcccaa acacctcttt ctgtgggtcc tgaccttggc
901  agttccaatg gctaaaaggt ccaggaaggt ttaggatggg agccctcctg ctgcccccag
961  gaggtttgca atgtcctttg tagcatatat cctgccacac agtatgtgct tcccagatgt
```

```
1021  ttacagaaca taatgtgaaa atttaggccc aaaccttcac ttccattcat tgctatagac
1081  aaacagtgtt tgaagtgtat gttgcctgct aggagtctga caatcaggcg ctttcctgaa
1141  tttaagcact ggtttgtttg taataggaag cttgggaaat gcctcttcct ctgctccagc
1201  ccctatctcc cctgtctggg ctgcatgcac ttcctgtgtg ggtaagggac ctcatggttc
1261  catattctga cgggaagccg gactgcaggc atctgatcct tttgactaaa tggaagaact
1321  atcccaacgg tccttagaaa cgggcttccc caggagcgat gtctgataat gtcctcctct
1381  gtgaggggct gcctaagagg tgtcggtgtt caagaaagca gggctcccag aaaagaagag
1441  gatggtggtg tgaggtgggg aaggctacac tctacacctt gcttctcaac tatcccctta
1501  ctggggtctt acgagattct ttttgtggtg tggagaggag agctgagtgg tcaagtctca
1561  ccactaacgg gttcaagcct tggcctcagt ccttggcttc ttcaggatta catcctagac
1621  ccaactctct ctgccatggg gactcccttg cctaacccca aaacatacca tttccccaga
1681  aaggaattag tattgctaat tggtgataat tgttcccaaa tagcccactg gtgaaaacaa
1741  agcctgattt cacctgactg ttacagattg gtcttaaggc ggtagacgtg agtgacatag
1801  gagtgacacc tcagggctca tcgtctgtgt ctgtggggtt cgttttcaga ggcaaaggct
1861  gctgtggcct ctgaggatga gcttgccgag agcccagaga tgacagagga acagctgctg
1921  gatagcttcc acctcatggc cccatcccgt gaggaccagc ccatcctgtg gaatgccatt
1981  agcacctaca gcagcatgcg ggcccgggag atcactgacc tcaagaaatg gaaggtgaga
2041  ccctgcactc agaccttcag gtttagctat ctacgtgaag aggtttgtct agacgatttc
2101  ttaaagggca ctgagcatgg ggctgagaac ggggatataa ctacccccat ccctgatgta
2161  tttctgcctc cttaaaaata tggcaagtat ctcagagcat aaggtaggcc atttttcagt
2221  ctaggtttct ctgtcaccga gtacgcacgt tcagtgattg ttagccacca accagctcca
2281  cggttttgcc agcctttagc tatgcacttt agctatgcag taaacttctc tagctttact
2341  ggctgttttt caacttgacc acttgggggа gacagagaac caaaggtgga gagaaagtac
2401  ggcagaggca ttgaagaagt acacctaagg aaatgaaagg ataaacattg ttaggggcac
2461  tttagaattt catatggaaa ttgtccaaat cagtgccttg ttccgtaatc aatttgacat
2521  acaccaaatg caaggatggc tgtttgaaaa atctaggcat ttatgatgct aaattccaca
2581  cacagagact gagcctgtct tttttattag agttcaggtg ctcaagttat tcagagatag
2641  ccagggtcag gaagcattta taccattggc caggctctta ccacaatgtc gttaaggggg
2701  tctccagaaa atgccactga gggaggatga gagtggtgtc cctgtccttt atctacatag
2761  cccaagccag agaccaacct gtcctgctca cagatgggga aacatctcag ccgttgtcta
2821  aattgataat ttttgtctct tgtactcatg ctaatataaa attatccttt taggagccct
2881  gccaacggga actctataaa gtgttagaga gattagctgc cgctcaacag aaagcaggag
2941  atgagatcta caaattttat ctgccaaact gcaacaagaa tggattttat cacagcaaac
3001  aggtaggtgg ctttgctcat ccagatcctt gtaaaacttc atgatttttt tttttaaagt
3061  caaatgattc acaggcccaa tacacatcat gggtagcttt cttaggtgag atccagccct
3121  gcagtagttg ggagaagcta gtcctgagaa agagatagtg tgatggatga ggaacacttc
3181  agccagaagg gaggactaag cattagtgtg atgagtgagg agcacttcag ttaacaggga
3241  ggactaagca ttagtgtgat gagtgaggac cacttcaagc cagagggagg actaacattg
3301  gcagtatgat gagtgaggag cacttcagcc agtagggagg actaaccatt agtctcatca
3361  ctaggagcac ctcagccaag tagggaggac taaccattag tctcacactc acccaacatc
3421  ttcagtcagg actaagcatt agtgtgatga gtgaggagca cttcagtcag tagggaggac
3481  tacattagtg tgatgagtga ggagcacttc agccagtagg gaggactaac cgttcactca
3541  gattagcaga gatggatgtt ccatatactg atgtccaggt ttcagttcct cacaactaga
3601  ggaaagggac acagtcagtg taggagacag atgtctcgcg ttctctcttc ccacaaataa
3661  aaacaaactc tgtagtaaga cacaccaatt gtgctttgcc tagcaataaa tgagattgaa
3721  gaagtccagg cttaatttcg acgcaacttt agaactcagg gaagtgcaag ttctggaatt
3781  tcattgagga aaaacttgag gtctaggtct agccgtgtgg tagagatggt gagacctatc
3841  gttgagctcc tttggcagag ggccatggag caggtaaccg tcaaaacaat ataccactga
3901  gtaaacagat gagattgtta tcaggtgtgc cataaagcca acctctccgt tttgtgatga
3961  caaccagaag ggcattggtc tgccgagcct tagccagcag gtagctgtgc agtgcttggc
4021  ctcactgagg gacagggtgg ccagagctct tacctcctgc tgctcttgac ctcggtcctg
4081  tctttgcagt gcgagacatc tctggatgga gaagctgggc tctgctggtg tgtctaccca
4141  tggagtggga agaagatccc tggatctctg gagaccagag gggaccccaa ctgccaccag
4201  tattttaatg tgcaaaactg aaagttgttt cctccctcct tcttcacaca aaatatttaa
4261  gtatatagtg tatttatact ccggagcaca ccattttata tatgtgtata tgtatatatc
4321  caggaactag tttttatact ccacatgctg cttgatgtac aagtgggttt gtatttattc
4381  actctaagtt tatttttttc taccctgtcc ttgtgctgta ttaatttata taactgaagc
4441  ttttctcatc tccatacatg taaatactac catctcagct cttccagagt tctgctttga
4501  aagggcagcg cggtacgtgc ctagaacgag cacaagtcag tctgaggtag gggcctttca
4561  gtgggttcag ggaggaaggt tagccctggc tcggggagac ttcctcatcg aatcccacag
4621  gtctgtgtct gatgcctatt ggctgggaag gttccgatgt tggttgtgta atcaaagcta
4681  aacgtggaaa gctgcgtccc atgcactgtt aaacacacgt ctggaataaa acattctacc
4741  tggaaacact gctgtctctg tggaattcca gctctgtgct cattccctca gtccgttcgg
```

```
4801  ctttcccgct cgcctgattc ctgggtctgt gctttgggga tagatgttgc aatacagggt
4861  gcttgtttgt ttacagaaca ccctggacaa acactctgtg actttatggt cccattttca
4921  agcagcatca ggcctctgtc tgggccagac tacagagccc ctcctccttg gtccatctcc
4981  ctttcttccc agggccctca g
```

## INJURYMARKER8

INJURYMARKER8 is 579 bp rat expressed sequence tag (EST) [AA851963]. The nucleic acid was initially identified in a cloned fragment having the following sequence:

```
1   TGTACATAATTTATTAAAAATGTCTCTGACACAAATAATGACTCCACTGCATACATAGTTGGTGTTCAAAAATTTCCCCA

81  ATGTTTGTTCTGGACACAATTGTTATTAGCCAACTCGGTGAATTCAAGACATTGTTCCACACAATGAACAATCGCACACA

161 TGAGAACTGCACCTAGAATGTCCATCCTAGAATCTCCATCCATCCAGTCAAAGTGCTGAGCTCACTGACTGAAGGAAACA

241 TGACCTGTGTTCTAGA  (SEQ ID NO: 13)
```

The cloned sequence was assembled into a contig resulting in the following consensus sequence:

```
1   TGTACATAATTTATTAAAAATGTCTCTGACACAAATAATGACTCCACTGCATACATAGTTGGTGTTCAAAAATTTCCCCA

81  ATGTTTGTTCTGGACACAATTGTTATAAGCCAACTCGGTGAATTCAAGACATTGTTCCACACAATGAACAATCGCACACA

161 TGAGAACTGCACCTAGAATGTCCATCCTAGAATCTCCATCCATCCAGTCAAAGTGCTGAGCTCACTGACTGAAGGAAACA

241 TGACCTGTGTTCTAGAACGTAGCTGGCTATGAAGTTTACTCATGTGTAAATTCCTTAAAAAGATTAAATTGTTTGGCCCA

321 TTTCTATATTTCATAAAATAACTATAATTACAAACTTTCTAAAAATAATTTTACAACCATGTAATTATGACTAACCATAT

401 CATCTAAAAAGTAAGTGAAGTCATTGTCCTAGAGATTGTCTGAGATTATTCTGCTGAGAAGCTTACTTCAAACTCTTATC

481 ACTACTTCCTACTTCCAGTGTCCTTGAATTAAGAACAGAAATTGTAACTATGCTATTCTACATCAGATTGACACAACCTA

561 CTTCTAAGTACACTATTGC  (SEQ ID NO: 14)
```

Blast-N Results:

**>gb:GENBANK-ID:AA851963|acc:AA851963 EST194732 Normalized rat spleen, Bento Soares Rattus sp. cDNA clone RSPAO86 3' end, mRNA sequence - Rattus sp., 538 bp (RNA).**

```
Top  Previous Match  Next Match
          Length = 538

  Plus Strand HSPs:

 Score = 2681 (402.3 bits), Expect = 8.1e-115, P = 8.1e-115
 Identities = 537/538 (99%), Positives = 537/538 (99%), Strand = Plus / Plus

Query:    42 CTCCACTGCATACATAGTTGGTGTTCAAAAATTTCCCCAATGTTTGTTCTGGACACAATT 101
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 CTCCACTGCATACATAGTTGGTGTTCAAAAATTTCCCCAATGTTTGTTCTGGACACAATT 60

Query:   102 GTTATAAGCCAACTCGGTGAATTCAAGACATTGTTCCACACAATGAACAATCGCACACAT 161
             |||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 GTTATTAGCCAACTCGGTGAATTCAAGACATTGTTCCACACAATGAACAATCGCACACAT 120

Query:   162 GAGAACTGCACCTAGAATGTCCATCCTAGAATCTCCATCCATCCAGTCAAAGTGCTGAGC 221
```

```
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 GAGAACTGCACCTAGAATGTCCATCCTAGAATCTCCATCCATCCAGTCAAAGTGCTGAGC 180

Query:   222 TCACTGACTGAAGGAAACATGACCTGTGTTCTAGAACGTAGCTGGCTATGAAGTTTACTC 281
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 TCACTGACTGAAGGAAACATGACCTGTGTTCTAGAACGTAGCTGGCTATGAAGTTTACTC 240

Query:   282 ATGTGTAAAATTCCTTAAAAAGATTAAATTGTTTGGCCCATTTCTATATTTCATAAAATAA 341
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   241 ATGTGTAAATTCCTTAAAAAGATTAAATTGTTTGGCCCATTTCTATATTTCATAAAATAA 300

Query:   342 CTATAATTACAAACTTTCTAAAAATAATTTTACAACCATGTAATTATGACTAACCATATC 401
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   301 CTATAATTACAAACTTTCTAAAAATAATTTTACAACCATGTAATTATGACTAACCATATC 360

Query:   402 ATCTAAAAAGTAAGTGAAGTCATTGTCCTAGAGATTGTCTGAGATTATTCTGCTGAGAAG 461
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   361 ATCTAAAAAGTAAGTGAAGTCATTGTCCTAGAGATTGTCTGAGATTATTCTGCTGAGAAG 420

Query:   462 CTTACTTCAAACTCTTATCACTACTTCCTACTTCCAGTGTCCTTGAATTAAGAACAGAAA 521
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   421 CTTACTTCAAACTCTTATCACTACTTCCTACTTCCAGTGTCCTTGAATTAAGAACAGAAA 480

Query:   522 TTGTAACTATGCTATTCTACATCAGATTGACACAACCTACTTCTAAGTACACTATTGC 579
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   481 TTGTAACTATGCTATTCTACATCAGATTGACACAACCTACTTCTAAGTACACTATTGC 538
```

## INJURYMARKER9

INJURYMARKER9 is a 2495 nucleotide catalese-encoding sequence[M11670], having the following nucleic acid sequence:

```
1     attgcctacc ccgggtggag accgtgctcg tccggccctc ttgcctcacg ttctgcagct
61    ctgcagctcc gcaatcctac accatggcgg acagccggga cccagccagc gaccagatga
121   agcagtggaa ggagcagcgg gcccctcaga aacccgatgt cctgaccacc ggaggcggga
181   acccaatagg agataaactt aatatcatga ctgcggggcc ccgagggccc ctcctcgttc
241   aagatgtggt tttcaccgac gagatggcac actttgacag agagcggatt cctgagagag
301   tggtacatgc aaagggagca ggtgcttttg gatactttga ggtcacccac gatattacca
361   gatactccaa ggcaaaggtg tttgagcata ttgggaagag gactcctatt gccgtccgat
421   tctccacagt cgctggagag tcaggctcag ctgacacagt tcgtgaccct cgtgggtttg
481   cagtgaaatt ctacactgaa gatggtaact gggacctcgt gggaaacaac acccctattt
541   tcttcatcag ggatgccatg ttgtttccat cctttatcca tagccagaag agaaacccac
601   aaactcacct gaaggaccct gacatggtct gggacttctg gagtctttgt ccagagtctc
661   tccatcaggt tactttcttg ttcagcgacc gagggattcc agatggacat cggcacatga
721   atggctatgg ctcacacacc ttcaagctgg ttaatgcgaa tggagaggca gtgtactgca
781   agttccatta caagactgac cagggcatca aaaacttgcc tgttgaagag gcaggaagac
841   ttgcacagga agacccggat tatggcctcc gagatctttt caatgccatc gccagtggca
901   attacccatc ctggactttt tacatccagg tcatgacttt caaggaggca gaaaccttcc
961   catttaatcc atttgacctg accaaggttt ggcctcacaa ggactaccct cttataccag
1021  ttggcaaact ggtcttaaac agaaatcctg ctaattattt tgctgaagtt gaacagatgg
1081  cttttgaccc aagcaacatg ccccctggca ttgagcccag cccggacaag atgctccagg
1141  gccgcctttt tgcttaccca gacactcacc gccaccgcct gggaccaaac tatctgcaga
1201  tacctgtgaa ctgtccctac cgtgctcgcg tggccaacta ccagcgcgat ggccccatgt
1261  gcatgcatga caaccagggt ggtgctccca actactaccc caacagcttc agcgcaccag
1321  agcagcaggg ctcggccctg gagcaccata gccagtgctc tgcagatgtg aagcgcttca
1381  acagtgctaa tgaagacaac gtcactcagg tgcggacatt ctatacgaag gtgttgaatg
1441  aggaggagag gaaacgcctg tgtgagaaca ttgccaacca cctgaaagat gctcagcttt
1501  tcattcagag gaaagcggtc aagaatttca ctgacgtcca ccctgactac ggggcccgag
1561  tccaggctct tctggaccag tacaactccc agaagcctaa gaatgcaatt cacacctacg
1621  tacaggccgg ctctcacata gctgccaagg gaaaagctaa cctgtaaagc acgggtgctc
1681  agcctcctca gcctgcactg aggagatccc tcatgaagca gggcacaagc ctcaccagta
1741  atcatcgctg gatggagtct cccctgctga agcgcagact cacgctgacg tctttaaaac
1801  gataatccaa gcttctagag tgaatgatag ccatgctttt gatgacattt cccgaggggg
1861  aaattaaaga ttagggctta gcaatcactt aacagaaaca tggatctgct taggacttct
1921  gtttggatta ttcatttaaa atgattacaa gaaaggtttt ctagccagaa acatgatttg
```

```
1981  attagatatg atatatgata aaatcttggt gattttacta tagtcttatg ttacctcaca
2041  gcctggtata tatacaacac acacacacac acacacacac acacaccaaa acacacatac
2101  actatacaca cacacacaca cacacactaa aacacacata cacaacacac acatacacta
2161  cacacacaga acacacaaca caaacataca cacataggca cacacacaca cacacacaca
2221  cacacacaca cacacacaca cacacatgaa tgaagggatt ataaagatgg cccacccaga
2281  atttttttt attttctaa ggtccttata agaaaaacca tacttggatc atgtcttcca
2341  aaaataactt tagcactgtt gaaacttaat gtttattcct gtgtagttga ttggattcct
2401  tttccccttg aaattatgtt tatgctgata cacagtgatt tcacataggg tgatttgtat
2461  ttgcttacat ttttacaata aaatgatctt catgg
```

**INJURYMARKER10**

INJURYMARKER10 is a 1884 nucleotide betaine homocysteine methyl transferase-encoding sequence [AF038870]:

```
1     caagcctttg ctggagaccg ctcctgtcca gtccgcagct ggcttcagcg ccactcagga
61    caccggaaag atggcaccga ttgccggcaa gaaggccaag aggggaatct tagaacgctt
121   aaatgctggc gaagtcgtga tcggagatgg gggatttgtc tttgcactgg aaaagagggg
181   ctacgtaaag gctggaccct ggaccccaga ggctgcggtg gagcaccccg aggcagttcg
241   gcagcttcat cgggagttcc tcagagctgg atcgaacgtc atgcagacct tcactttcta
301   tgcaagtgag gacaagctgg aaaaccgagg gaactacgtg gcagagaaga tatctgggca
361   gaaggtcaat gaagctgctt gtgacattgc acggcaagtt gctgacgaag gggatgcatt
421   ggttgcagga ggtgtgagtc agacaccttc ctacctcagc tgcaagagtg agacggaagt
481   taaaaagata tttcaccaac agcttgaggt cttcatgaag aagaatgtgg acttcctcat
541   tgcagagtat tttgaacatg ttgaagaagc cgtgtgggca gtcgaggcct taaaaacatc
601   cgggaagcct atagcggcta ccatgtgcat cggacctgaa ggagatctac atggcgtgtc
661   tcctggagag tgcgcagtgc gtttggtaaa agcaggtgcc gccattgtcg gtgtgaactg
721   ccacttcgac cccagcacca gcttgcagac aataaagctc atgaaggagg gtctggaagc
781   agctcggctg aaggcttact tgatgagcca cgccctggcc taccacaccc ctgactgtgg
841   caaacaggga tttattgatc tcccagaatt ccccttgga ttggaaccca gagttgccac
901   cagatgggat attcaaaaat acgccagaga ggcctacaac ctgggggtca ggtacattgg
961   cggctgctgc ggatttgagc cctaccacat cagggccatt gcagaggagc tcgccccaga
1021  aaggggattt ttaccaccag cttcagaaaa acatggcagc tggggaagtg gtttggacat
1081  gcacaccaaa ccctggatca gggcaagggc caggaaagaa tactggcaga atcttcgaat
1141  agcttcgggc agaccgtaca atccttcgat gtccaagccg gatgcttggg gagtgacgaa
1201  aggggcagca gagctgatgc agcagaagga agccaccact gagcagcagc tgagagcgct
1261  cttcgaaaaa caaaaattca aatccgcaca gtagccacag gccagcggtt cggggcgaat
1321  tcctccaggt ccgggccaca gtgtgcaccc ggaaggagaa ggcatctcta aaccagcgtt
1381  tgtgttgatg ccggcttaca cctgtgattg gtgctagtta gacaaaatgg agtcacagat
1441  agcatttcac agttacaaaa ctacgcttta gaattttacc tagaaggaag aaaggagaag
1501  tccacagtaa atcctgaaca catttcctac gtgcctgtcg cattacaggc gcacaggagt
1561  cactgcagcg aagagaaagt cacccgacgt caatctcatt tcagataggg ggataggaca
1621  ccacctccac gagtgacata gaaccattca gggaccgtat cataagtgac acagcaacca
1681  tctatatcta agatgcttcc caagtggatt ccaagatctt ttgagcagga cccttaggca
1741  gaaacaacac acaccagccc tgtaaaactt aacagataac tgatccattc tgtaattctg
1801  taatctctgt tctgactgct tccattccat ttcattaata aaaacatgcc ggttgaaaac
1861  cttcaaaaaa aaaaaaaaaa aaaa
```

Principle components analysis was used to generate three eigenvectors used to transform the original expression level data matrix, as shown in Table 6 below. Eigenvector 1 values represent NSAIDs associated with hepatoxicity involving hepatocellular damage, Eigenvector 2 values represent NSAIDs associated with hepatoxicity involving cholestasis,

and Eigenvector 3 values represent NSAIDs associated with hepatoxicity involving elevated transaminase level.

Table 6: Transform Eigenvectors for Hepatoxicity by Injury Type

| Gene | Eigenvector 1 | Eigenvector 2 | Eigenvector3 |
|---|---|---|---|
| INJURYMARKER1 | 58.7 | 0.325 | -15.2 |
| INJURYMARKER2 | 20.5 | -3.23 | 3.01 |
| INJURYMARKER3 | -16.9 | -6.52 | -2.09 |
| INJURYMARKER4 | -10.3 | 0.351 | -1.45 |
| INJURYMARKER5 | -7.59 | -0.152 | -0.310 |
| INJURYMARKER6 | 11.4 | -2.69 | 2.49 |
| INJURYMARKER7 | -16.0 | -1.57 | 8.71 |
| INJURYMARKER8 | -11.6 | 1.13 | 5.36 |
| INJURYMARKER9 | -11.0 | -0.351 | 0.078 |
| INJURYMARKER10 | 7.55 | 0.618 | 4.65 |
| % of variation explained | 99.0 | 0.7 | 0.3 |

These eigenvectors may be used to transform the expression levels of INJURYMARKERS 1-10 ("INJURYMARKERS") in response to a given drug, in order to predict that drug's hepatotoxicity injury type. For example, expression levels of INJURYMARKERS correlating with Eigenvector 1 indicates that the test drug has a risk of hepatotoxicity involving hepatocellular damage. Alternatively, a drug's INJURYMARKERS expression profile can be generated simultaneously with the above-described training set (or an equivalent set) run in parallel with the test drug, and expression levels associated with the test drug directly compared to those of the training set.

## GENERAL METHODS

The RISKMARKER (*i.e.* RISKMARKERS 1-8) and INJURYMARKER (*i.e.* INJURYMARKERS 1-10) nucleic acids and encoded polypeptides can be identified using the information provided above. In some embodiments, the RISKMARKER or INJURYMARKER nucleic acids and polypeptide correspond to nucleic acids or polypeptides which include the various sequences (referenced by SEQ ID NOs) disclosed for each RISKMARKER or INJURYMARKER polypeptide.

In its various aspects and embodiments, the invention includes providing a test cell population which includes at least one cell that is capable of expressing one or more of the sequences RISKMARKER 1-8 or INJURYMARKER 1-10. By "capable of expressing" is meant that the gene is present in an intact form in the cell and can be expressed. Expression of

one, some, or all of the RISKMARKER or INJURYMARKER sequences is then detected, if present, and, preferably, measured. Using sequence information provided by the database entries for the known sequences, or the sequence information for the newly described sequences, expression of the RISKMARKER or INJURYMARKER sequences can be detected (if present) and measured using techniques well known to one of ordinary skill in the art. For example, sequences within the sequence database entries corresponding to RISKMARKER or INJURYMARKER sequences, or within the sequences disclosed herein, can be used to construct probes for detecting RISKMARKER or INJURYMARKER RNA sequences in, *e.g.*, northern blot hybridization analyses or methods which specifically, and, preferably, quantitatively amplify specific nucleic acid sequences. As another example, the sequences can be used to construct primers for specifically amplifying the RISKMARKER or INJURYMARKER sequences in, *e.g.*, amplification-based detection methods such as reverse-transcription based polymerase chain reaction. When alterations in gene expression are associated with gene amplification or deletion, sequence comparisons in test and reference populations can be made by comparing relative amounts of the examined DNA sequences in the test and reference cell populations.

Expression can be also measured at the protein level, *i.e.*, by measuring the levels of polypeptides encoded by the gene products described herein. Such methods are well known in the art and include, *e.g.*, immunoassays based on antibodies to proteins encoded by the genes.

Expression level of one or more of the RISKMARKER or INJURYMARKER sequences in the test cell population, *e.g.* rat hepatocytes, is then compared to expression levels of the sequences in one or more cells from a reference cell population. Expression of sequences in test and control populations of cells can be compared using any art-recognized method for comparing expression of nucleic acid sequences. For example, expression can be compared using GENECALLING® methods as described in US Patent No. 5,871,697 and in Shimkets et al., Nat. Biotechnol. 17:798-803.

In various embodiments, the expression of one or more sequences which are markers of hepatoxicity risk, *i.e.* RISKMARKERS 1-8, is compared. In other embodiments, the expression of one or more sequences which are markers of hepatoxicity injury type, *i.e.* INJURYMARKERS, is compared. In further embodiments, expression of one or more RISKMARKERS and INJURYMARKERS may be compared to predict both hepatoxicity risk and type of hepatoxicity injury.

In various embodiments, the expression of 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or all of the sequences represented by RISKMARKER 1-8 and INJURYMARKER 1-10 are measured. If desired, expression of these sequences can be measured along with other sequences whose expression is known to be altered according to one of the herein described parameters or conditions.

The reference cell population includes one or more cells for which the compared parameter is known. The compared parameter can be, *e.g.*, hepatotoxic agent expression status. By "hepatotoxic agent expression status" is meant that it is known whether the reference cell has had contact with a hepatotoxic agent. An example of a hepatotoxic agent is, *e.g.*, a thiazolidinedione such as troglitazone. Whether or not comparison of the gene expression profile in the test cell population to the reference cell population reveals the presence, or degree, of the measured parameter depends on the composition of the reference cell population. For example, if the reference cell population is composed of cells that have not been treated with a known hepatotoxic agent, a similar gene expression level in the test cell population and a reference cell population indicates the test agent is not a hepatotoxic agent. Conversely, if the reference cell population is made up of cells that have been treated with a hepatotoxic agent, a similar gene expression profile between the test cell population and the reference cell population indicates the test agent is a hepatotoxic agent.

In various embodiments, a RISKMARKER or INJURYMARKER sequence in a test cell population is considered comparable in expression level to the expression level of the RISKMARKER or INJURYMARKER sequence if its expression level varies within a factor of 2.0, 1.5, or 1.0 fold to the level of the RISKMARKER or INJURYMARKER transcript in the reference cell population. In various embodiments, a RISKMARKER or INJURYMARKER sequence in a test cell population can be considered altered in levels of expression if its expression level varies from the reference cell population by more than 1.0, 1.5, 2.0 or more fold from the expression level of the corresponding RISKMARKER or INJURYMARKER sequence in the reference cell population.

Alternatively, the absolute expression level matrix of the 8 RISKMARKER and/or 10 INJURYMARKER fragments in a test cell can be transformed using the principal component eigenvectors described above, or similar eigenvalues generated from parallel dosed members of the training set as internal controls. The expression eigenvalues for the test cell can then be compared to the training set eigenvalues described herein, or a parallel-run training set, if any.

The RISKMARKER expression level combination is considered similar to Low Risk idiosyncratic NSAIDS (several of which have been withdrawn), if the test drug's expression profile is within the 95% confidence interval (CI) of the centroid of that risk class. *See* Table 4. The test drug is considered Very Low Risk idiosyncratic if the transformed expression profile falls within the 95% CI of the centroid of that class. The test drug is considered Overdose Risk if the expression profile falls within the 95% CI of the centroid of that class. If the compound fails to associate with any of these compounds it will be categorized as unclassifiable.

Similarly, the INJURYMARKER expression level combination is considered indicative of hepatocellular damage induced by idiosyncratic NSAIDS, if the test drug's expression profile is within the 95% confidence interval (CI) of the centroid of that injury type. *See* Table 6. The test drug is considered to induce idiosyncratic cholestasis if the transformed expression profile falls within the 95% CI of the centroid of that injury type. The test drug is considered to induce elevated transaminase level if the expression profile falls within the 95% CI of the centroid of that class. If the compound fails to associate with any of these compounds it will be categorized as unclassifiable.

If desired, comparison of differentially expressed sequences between a test cell population and a reference cell population can be done with respect to a control nucleic acid whose expression is independent of the parameter or condition being measured. Expression levels of the control nucleic acid in the test and reference nucleic acid can be used to normalize signal levels in the compared populations.

In some embodiments, the test cell population is compared to multiple reference cell populations. Each of the multiple reference populations may differ in the known parameter. Thus, a test cell population may be compared to a first reference cell population known to have been exposed to a hepatotoxic agent, as well as a second reference population known have not been exposed to a hepatotoxic agent.

The test cell population that is exposed to, *i.e.*, contacted with, the test hepatotoxic agent can be any number of cells, *i.e.*, one or more cells, and can be provided *in vitro, in vivo,* or *ex vivo.*

In other embodiments, the test cell population can be divided into two or more subpopulations. The subpopulations can be created by dividing the first population of cells to create as identical a subpopulation as possible. This will be suitable, in, for example, *in vitro* or *ex vivo* screening methods. In some embodiments, various sub populations can be exposed

to a control agent, and/or a test agent, multiple test agents, or, *e.g.*, varying dosages of one or multiple test agents administered together, or in various combinations.

Preferably, cells in the reference cell population are derived from a tissue type as similar as possible to test cell, *e.g.*, liver tissue. In some embodiments, the control cell is derived from the same subject as the test cell, *e.g.*, from a region proximal to the region of origin of the test cell. In other embodiments, the reference cell population is derived from a plurality of cells. For example, the reference cell population can be a database of expression patterns from previously tested cells for which one of the herein-described parameters or conditions (hepatotoxic agent expression status is known.

The test agent can be a compound not previously described or can be a previously known compound but which is not known to be a hepatotoxic agent.

The subject is preferably a mammal. The mammal can be, *e.g.*, a human, non-human primate, mouse, rat, dog, cat, horse, or cow.

## SCREENING FOR TOXIC AGENTS

In one aspect, the invention provides a method of identifying toxic agents, *e.g.*, hepatotoxic agents. The hepatotoxic agent can be identified by providing a cell population that includes cells capable of expressing one or more nucleic acid sequences homologous to those of RISKMARKER 1-8 or INJURYMARKER 1-10. The sequences need not be identical to sequences including RISKMARKER or INJURYMARKER nucleic acid sequences, as long as the sequence is sufficiently similar that specific hybridization can be detected. Preferably, the cell includes sequences that are identical, or nearly identical to those identifying the RISKMARKER or INJURYMARKER nucleic acids described herein.

Expression of the nucleic acid sequences in the test cell population is then compared to the expression of the nucleic acid sequences in a reference cell population, which is a cell population that has not been exposed to the test agent, or, in some embodiments, a cell population exposed the test agent. Comparison can be performed on test and reference samples measured concurrently or at temporally distinct times. An example of the latter is the use of compiled expression information, *e.g.*, a sequence database, which assembles information about expression levels of known sequences following administration of various agents. For example, alteration of expression levels following administration of test agent can be compared to the expression changes observed in the nucleic acid sequences following administration of a control agent, *e.g.* a NSAID such as ketoprofen.

An alteration in expression of the nucleic acid sequence in the test cell population compared to the expression of the nucleic acid sequence in the reference cell population that has not been exposed to the test agent indicates the test agent is a hepatotoxic agent.

The invention also includes a hepatotoxic agent identified according to this screening method.

In some embodiments of the method of the invention, the test agent is an idiosyncratic hepatotoxic agent, *e.g.* a NSAID, and the reference agent is also a NSAID. As described above, RISKMARKER (*e.g.* RISKMARKERS 1-8) expression level patterns can be used to predict the level of hepatoxicity risk (*i.e.* low, very low, or overdose) associated with a given test agent, *e.g.* a NSAID. In one embodiment, the reference NSAID (*i.e.* used with the reference cell population) is a NSAID classified as having a low risk of hepatoxicity. The test agent is then identified as having a low risk of hepatoxicity if no qualitative difference in expression levels is identified in comparison to expression levels in the reference population exposed to a low risk NSAID. In certain embodiments, the low risk NSAID is Benoxaprofen, Bromfenac, Diclofenac, Phenylbutazone, or Sulindac. In another embodiment, the reference NSAID is a NSAID classified as having a very low risk of hepatoxicity. The test agent is then identified as having a very low risk of hepatoxicity if no qualitative difference in expression levels is identified in comparison to expression levels in the reference population exposed to a very low risk NSAID. In certain embodiments, the very low risk NSAID is Etodolac, Fenoprofen, Flurbiprofen, Ibuprofen, Indomethacin, Ketoprofen, Meclofenamate, Mefenamic Acid, Nabumetone, Naproxen, Oxaprozin, Piroxicam, Suprofen, Tenoxicam, Tolmentin, or Zomepirac. In still another embodiment, the reference NSAID is a NSAID classified as having an overdose risk of hepatoxicity. The test agent is then identified as having an overdose risk of hepatoxicity if no qualitative difference in expression levels is identified in comparison to expression levels in the reference population exposed to an overdose risk NSAID. In certain embodiments, the overdose risk NSAID is Acetaminophen, Acetylsalicylic acid, or Phenacetin. In some embodiments, the difference in expression levels is determined by comparing expression transformation eigenvectors (for risk class) for the test cell and reference cell populations, as described above.

As also described above, INJURYMARKER (*e.g.* INJURYMARKERS 1-10) expression level patterns can be used to predict the type of hepatoxicity injury (*i.e.* hepatocellular damage, cholestasis, or elevated transaminase level) associated with a given test agent, *e.g.* a NSAID. In some embodiments, the reference NSAID is a NSAID classified as

inducing hepatocellular damage. The test agent is then identified as likely to induce hepatocellular damage if no qualitative difference in expression levels is identified in comparison to expression levels in the reference population exposed to a NSAID which induces hepatocellular damage. In certain embodiments, the hepatocellular damage inducing NSAID is Acetaminophen, Flurbiprofen, or Ketoprofen. In another embodiment, the reference NSAID is a NSAID classified as inducing cholestasis. The test agent is then identified as likely to induce cholestasis if no qualitative difference in expression levels is identified in comparison to expression levels in the reference population exposed to a NSAID which induces cholestasis. In certain embodiments, the cholestatis-inducing NSAID is Benoxaprofen, Nabumetone, or Sulindac. In yet another embodiment, the reference NSAID is a NSAID classified as inducing elevated transaminase level. The test agent is then identified as likely to induce elevated transaminase level if no qualitative difference in expression levels is identified as compared to expression levels in the reference population exposed to a NSAID which induces elevated transaminase levels. In certain embodiments, the elevated transaminase level inducing NSAID is Zomepirac, Mefenamic acid, or Tenoxicam. In some embodiments, the difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations, as desribed above.

## ASSESSING TOXICITY OF A TOXIC AGENT IN A SUBJECT

The differentially expressed RISKMARKER or INJURYMARKER sequences identified herein also allow for the hepatotoxicity of a hepatotoxic agent to be determined or monitored. In this method, a test cell population from a subject is exposed to a test agent, *i.e.* a. hepatotoxic agent. If desired, test cell populations can be taken from the subject at various time points before, during, or after exposure to the test agent. Expression of one or more of the RISKMARKER or INJURYMARKER sequences in the cell population is then measured and compared to a reference cell population which includes cells whose hepatotoxic agent expression status is known. Preferably, the reference cells not been exposed to the test agent.

If the reference cell population contains no cells exposed to the treatment, a similarity in expression between RISKMARKER or INJURYMARKER sequences in the test cell population and the reference cell population indicates that the treatment is non-hepatotoxic. However, a difference in expression between RISKMARKER or INJURYMARKER sequences in the test population and this reference cell population indicates the treatment is hepatotoxic.

By "hepatotoxicity" is meant that the agent is damaging or destructive to liver when administered to a subject leads to liver damage.

As described in detail above, RISKMARKER expression patterns can be used to predict the level of hepatotoxicity risk (*e.g.* low risk, very low risk, overdose risk) associated with a test agent or drug, by comparison to RISKMARKER expression levels for reference drugs, *e.g.* NSAIDs, with a given classification of risk (*e.g.* very low risk). Similarly, INJURYMARKER expression patterns can be used to predict the type of hepatotoxicity damage (*e.g.* hepatocellular damage, cholestasis, elevated transaminase level) associated with a test agent or drug, by comparison to INJURYMARKER expression levels for reference drugs, *e.g.* NSAIDs, which induce a given type of hepatotoxic damage (*e.g.* cholestasis).

**RISKMARKER NUCLEIC ACIDS**

Also provided in the invention are novel nucleic acid comprising a nucleic acid sequence selected from the group consisting of RISKMARKER 1, and RISKMARKERS 6-8, or their complements, as well as vectors and cells including these nucleic acids.

Thus, one aspect of the invention pertains to isolated RISKMARKER nucleic acid molecules that encode RISKMARKER proteins or biologically active portions thereof. Also included are nucleic acid fragments sufficient for use as hybridization probes to identify RISKMARKER-encoding nucleic acids (*e.g.*, RISKMARKER mRNA) and fragments for use as polymerase chain reaction (PCR) primers for the amplification or mutation of RISKMARKER nucleic acid molecules. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (*e.g.*, cDNA or genomic DNA), RNA molecules (*e.g.*, mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments and homologs thereof. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

"Probes" refer to nucleic acid sequences of variable length, preferably between at least about 10 nucleotides (nt) or as many as about, *e.g.*, 6,000 nt, depending on use. Probes are used in the detection of identical, similar, or complementary nucleic acid sequences. Longer length probes are usually obtained from a natural or recombinant source, are highly specific and much slower to hybridize than oligomers. Probes may be single- or double-stranded and designed to have specificity in PCR, membrane-based hybridization technologies, or ELISA-like technologies.

An "isolated" nucleic acid molecule is one that is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Examples of isolated nucleic acid molecules include, but are not limited to, recombinant DNA molecules contained in a vector, recombinant DNA molecules maintained in a heterologous host cell, partially or substantially purified nucleic acid molecules, and synthetic DNA or RNA molecules. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated RISKMARKER nucleic acid molecule can contain less than about 50 kb, 25 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention, *e.g.*, a nucleic acid molecule having the nucleotide sequence of any of RISKMARKER 1, or RISKMARKER 6-8, or a complement of any of these nucleotide sequences, can be isolated using standard molecular biology techniques and the sequence information provided herein. Using all or a portion of these nucleic acid sequences as a hybridization probe, RISKMARKER or INJURYMARKER nucleic acid sequences can be isolated using standard hybridization and cloning techniques (*e.g.*, as described in Sambrook *et al.*, eds., Molecular Cloning: A Laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989; and Ausubel, *et al.*, eds., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1993.)

A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides corresponding to RISKMARKER nucleotide sequences can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer.

As used herein, the term "oligonucleotide" refers to a series of linked nucleotide residues, which oligonucleotide has a sufficient number of nucleotide bases to be used in a PCR reaction. A short oligonucleotide sequence may be based on, or designed from, a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar or complementary DNA or RNA in a particular cell or tissue.

Oligonucleotides comprise portions of a nucleic acid sequence having at least about 10 nt and as many as 50 nt, preferably about 15 nt to 30 nt. They may be chemically synthesized and may be used as probes.

In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule that is a complement of the nucleotide sequence shown in RISKMARKER 1, or RISKMARKER 6-8 . In another embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule that is a complement of the nucleotide sequence shown in any of these sequences, or a portion of any of these nucleotide sequences. A nucleic acid molecule that is complementary to the nucleotide sequence shown in RISKMARKER 1, or RISKMARKER 6-8 is one that is sufficiently complementary to the nucleotide sequence shown, such that it can hydrogen bond with little or no mismatches to the nucleotide sequences shown, thereby forming a stable duplex.

As used herein, the term "complementary" refers to Watson-Crick or Hoogsteen base pairing between nucleotides units of a nucleic acid molecule, and the term "binding" means the physical or chemical interaction between two polypeptides or compounds or associated polypeptides or compounds or combinations thereof. Binding includes ionic, non-ionic, Von der Waals, hydrophobic interactions, etc. A physical interaction can be either direct or indirect. Indirect interactions may be through or due to the effects of another polypeptide or compound. Direct binding refers to interactions that do not take place through, or due to, the effect of another polypeptide or compound, but instead are without other substantial chemical intermediates.

Moreover, the nucleic acid molecule of the invention can comprise only a portion of the nucleic acid sequence of RISKMARKER 1, or RISKMARKER 6-8 , *e.g.*, a fragment that can be used as a probe or primer or a fragment encoding a biologically active portion of RISKMARKER. Fragments provided herein are defined as sequences of at least 6 (contiguous) nucleic acids or at least 4 (contiguous) amino acids, a length sufficient to allow for specific hybridization in the case of nucleic acids or for specific recognition of an epitope in the case of amino acids, respectively, and are at most some portion less than a full length sequence. Fragments may be derived from any contiguous portion of a nucleic acid or amino acid sequence of choice. Derivatives are nucleic acid sequences or amino acid sequences formed from the native compounds either directly or by modification or partial substitution. Analogs are nucleic acid sequences or amino acid sequences that have a structure similar to, but not identical to, the native compound but differs from it in respect to certain components

or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type.

Derivatives and analogs may be full length or other than full length, if the derivative or analog contains a modified nucleic acid or amino acid, as described below. Derivatives or analogs of the nucleic acids or proteins of the invention include, but are not limited to, molecules comprising regions that are substantially homologous to the nucleic acids or proteins of the invention, in various embodiments, by at least about 45%, 50%, 70%, 80%, 95%, 98%, or even 99% identity (with a preferred identity of 80-99%) over a nucleic acid or amino acid sequence of identical size or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, or whose encoding nucleic acid is capable of hybridizing to the complement of a sequence encoding the aforementioned proteins under stringent, moderately stringent, or low stringent conditions. See *e.g.* Ausubel, *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1993, and below. An exemplary program is the Gap program (Wisconsin Sequence Analysis Package, Version 8 for UNIX, Genetics Computer Group, University Research Park, Madison, WI) using the default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2: 482-489, which in incorporated herein by reference in its entirety).

A "homologous nucleic acid sequence" or "homologous amino acid sequence," or variations thereof, refer to sequences characterized by a homology at the nucleotide level or amino acid level as discussed above. Homologous nucleotide sequences encode those sequences coding for isoforms of a RISKMARKER polypeptide. Isoforms can be expressed in different tissues of the same organism as a result of, for example, alternative splicing of RNA. Alternatively, isoforms can be encoded by different genes. In the present invention, homologous nucleotide sequences include nucleotide sequences encoding for a RISKMARKER polypeptide of species other than humans, including, but not limited to, mammals, and thus can include, *e.g.*, mouse, rat, rabbit, dog, cat cow, horse, and other organisms. Homologous nucleotide sequences also include, but are not limited to, naturally occurring allelic variations and mutations of the nucleotide sequences set forth herein. A homologous nucleotide sequence does not, however, include the nucleotide sequence encoding a human RISKMARKER protein. Homologous nucleic acid sequences include those nucleic acid sequences that encode conservative amino acid substitutions (see below) in a RISKMARKER polypeptide, as well as a polypeptide having a RISKMARKER activity. A

homologous amino acid sequence does not encode the amino acid sequence of a human RISKMARKER polypeptide.

The nucleotide sequence determined from the cloning of human RISKMARKER genes allows for the generation of probes and primers designed for use in identifying and/or cloning RISKMARKER homologues in other cell types, *e.g.*, from other tissues, as well as RISKMARKER homologues from other mammals. The probe/primer typically comprises a substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, 25, 50, 100, 150, 200, 250, 300, 350 or 400 consecutive sense strand nucleotide sequence of a nucleic acid comprising a RISKMARKER sequence, or an anti-sense strand nucleotide sequence of a nucleic acid comprising a RISKMARKER sequence, or of a naturally occurring mutant of these sequences.

Probes based on human RISKMARKER nucleotide sequences can be used to detect transcripts or genomic sequences encoding the same or homologous proteins. In various embodiments, the probe further comprises a label group attached thereto, *e.g.*, the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can be used as a part of a diagnostic test kit for identifying cells or tissue which misexpress a RISKMARKER protein, such as by measuring a level of a RISKMARKER-encoding nucleic acid in a sample of cells from a subject *e.g.*, detecting RISKMARKER mRNA levels or determining whether a genomic RISKMARKER gene has been mutated or deleted.

"A polypeptide having a biologically active portion of RISKMARKER" refers to polypeptides exhibiting activity similar, but not necessarily identical to, an activity of a polypeptide of the present invention, including mature forms, as measured in a particular biological assay, with or without dose dependency. A nucleic acid fragment encoding a "biologically active portion of RISKMARKER" can be prepared by isolating a portion of RISKMARKER 1, or RISKMARKER 6-8, that encodes a polypeptide having a RISKMARKER biological activity, expressing the encoded portion of RISKMARKER protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of RISKMARKER. For example, a nucleic acid fragment encoding a biologically active portion of a RISKMARKER polypeptide can optionally include an ATP-binding domain. In another embodiment, a nucleic acid fragment encoding a biologically active portion of RISKMARKER includes one or more regions.

**RISKMARKER AND INJURYMARKER VARIANTS**

The invention further encompasses nucleic acid molecules that differ from the disclosed or referenced RISKMARKER or INJURYMARKER nucleotide sequences due to degeneracy of the genetic code. These nucleic acids thus encode the same RISKMARKER or INJURYMARKER protein as that encoded by nucleotide sequence comprising a RISKMARKER or INJURYMARKER nucleic acid as shown in, *e.g.*, RISKMARKER 1-8 INJURYMARKER 1-10.

In addition to the rat RISKMARKER or INJURYMARKER nucleotide sequence shown in RISKMARKER or INJURYMARKER 1, and RISKMARKER or INJURYMARKER 6-8, it will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of a RISKMARKER or INJURYMARKER polypeptide may exist within a population (*e.g.*, the human population). Such genetic polymorphism in the RISKMARKER or INJURYMARKER gene may exist among individuals within a population due to natural allelic variation. As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a RISKMARKER or INJURYMARKER protein, preferably a mammalian RISKMARKER or INJURYMARKER protein. Such natural allelic variations can typically result in 1-5% variance in the nucleotide sequence of the RISKMARKER or INJURYMARKER gene. Any and all such nucleotide variations and resulting amino acid polymorphisms in RISKMARKER or INJURYMARKER that are the result of natural allelic variation and that do not alter the functional activity of RISKMARKER or INJURYMARKER are intended to be within the scope of the invention.

Moreover, nucleic acid molecules encoding RISKMARKER or INJURYMARKER proteins from other species, and thus that have a nucleotide sequence that differs from the human sequence of RISKMARKER OR INJURYMARKER, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and homologues of the RISKMARKER or INJURYMARKER DNAs of the invention can be isolated based on their homology to the human RISKMARKER or INJURYMARKER nucleic acids disclosed herein using the human cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. For example, a soluble human RISKMARKER or INJURYMARKER DNA can be isolated based on its homology to human membrane-bound RISKMARKER or INJURYMARKER. Likewise, a membrane-bound human RISKMARKER or INJURYMARKER DNA can be isolated based on its homology to soluble human RISKMARKER or INJURYMARKER.

Accordingly, in another embodiment, an isolated nucleic acid molecule of the invention is at least 6 nucleotides in length and hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of RISKMARKER 1, or RISKMARKER 6-8. In another embodiment, the nucleic acid is at least 10, 25, 50, 100, 250 or 500 nucleotides in length. In another embodiment, an isolated nucleic acid molecule of the invention hybridizes to the coding region. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other.

Homologs (*i.e.*, nucleic acids encoding RISKMARKER or INJURYMARKER proteins derived from species other than human) or other related sequences (*e.g.*, paralogs) can be obtained by low, moderate or high stringency hybridization with all or a portion of the particular human sequence as a probe using methods well known in the art for nucleic acid hybridization and cloning.

As used herein, the phrase "stringent hybridization conditions" refers to conditions under which a probe, primer or oligonucleotide will hybridize to its target sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures than shorter sequences. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Since the target sequences are generally present at excess, at Tm, 50% of the probes are occupied at equilibrium. Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes, primers or oligonucleotides (e.g., 10 nt to 50 nt) and at least about 60°C for longer probes, primers and oligonucleotides. Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

Stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Preferably, the conditions are such that sequences at least about 65%, 70%, 75%, 85%, 90%, 95%, 98%, or 99% homologous to each other typically remain hybridized to each other. A non-limiting example of stringent hybridization conditions is hybridization in a high salt buffer comprising

6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 mg/ml denatured salmon sperm DNA at 65°C. This hybridization is followed by one or more washes in 0.2X SSC, 0.01% BSA at 50°C. An isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the sequence of RISKMARKER 1, or RISKMARKER 6-8 corresponds to a naturally occurring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (*e.g.*, encodes a natural protein).

In a second embodiment, a nucleic acid sequence that is hybridizable to the nucleic acid molecule comprising the nucleotide sequence of RISKMARKER 1, or RISKMARKER 6-8, or fragments, analogs or derivatives thereof, under conditions of moderate stringency is provided. A non-limiting example of moderate stringency hybridization conditions are hybridization in 6X SSC, 5X Denhardt's solution, 0.5% SDS and 100 mg/ml denatured salmon sperm DNA at 55°C, followed by one or more washes in 1X SSC, 0.1% SDS at 37°C. Other conditions of moderate stringency that may be used are well known in the art. See, *e.g.*, Ausubel *et al.* (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY, and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a third embodiment, a nucleic acid that is hybridizable to the nucleic acid molecule comprising the nucleotide sequence of RISKMARKER 1, or RISKMARKER 6-8, or fragments, analogs or derivatives thereof, under conditions of low stringency, is provided. A non-limiting example of low stringency hybridization conditions are hybridization in 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 mg/ml denatured salmon sperm DNA, 10% (wt/vol) dextran sulfate at 40°C, followed by one or more washes in 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS at 50°C. Other conditions of low stringency that may be used are well known in the art (*e.g.*, as employed for cross-species hybridizations). See, *e.g.*, Ausubel *et al.* (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY, and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY; Shilo et al., 1981, *Proc Natl Acad Sci USA 78*: 6789-6792.

## CONSERVATIVE MUTATIONS

In addition to naturally-occurring allelic variants of the RISKMARKER sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced into an RISKMARKER nucleic acid or directly into an RISKMARKER

polypeptide sequence without altering the functional ability of the RISKMARKER protein. In some embodiments, the nucleotide sequence of RISKMARKER 1, or RISKMARKER 6-8 will be altered, thereby leading to changes in the amino acid sequence of the encoded RISKMARKER protein. For example, nucleotide substitutions that result in amino acid substitutions at various "non-essential" amino acid residues can be made in the sequence of RISKMARKER 1, or RISKMARKER 6-8. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of RISKMARKER without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the RISKMARKER proteins of the present invention, are predicted to be particularly unamenable to alteration.

In addition, amino acid residues that are conserved among family members of the RISKMARKER proteins of the present invention, are also predicted to be particularly unamenable to alteration. As such, these conserved domains are not likely to be amenable to mutation. Other amino acid residues, however, (*e.g.*, those that are not conserved or only semi-conserved among members of the RISKMARKER proteins) may not be essential for activity and thus are likely to be amenable to alteration.

Another aspect of the invention pertains to nucleic acid molecules encoding RISKMARKER proteins that contain changes in amino acid residues that are not essential for activity. Such RISKMARKER proteins differ in amino acid sequence from the amino acid sequences of polypeptides encoded by nucleic acids containing RISKMARKER 1, or RISKMARKER 6-8, yet retain biological activity. In one embodiment, the isolated nucleic acid molecule comprises a nucleotide sequence encoding a protein, wherein the protein comprises an amino acid sequence at least about 45% homologous, more preferably 60%, and still more preferably at least about 70%, 80%, 90%, 95%, 98%, and most preferably at least about 99% homologous to the amino acid sequence of the amino acid sequences of polypeptides encoded by nucleic acids comprising RISKMARKER 1, or RISKMARKER 6-8.

An isolated nucleic acid molecule encoding a RISKMARKER protein homologous to can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of a nucleic acid comprising RISKMARKER 1, or RISKMARKER 6-8, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein.

Mutations can be introduced into a nucleic acid comprising RISKMARKER 1, or RISKMARKER 6-8 by standard techniques, such as site-directed mutagenesis and

PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in RISKMARKER is replaced with another amino acid residue from the same side chain family. Alternatively, in another embodiment, mutations can be introduced randomly along all or part of a RISKMARKER coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for RISKMARKER biological activity to identify mutants that retain activity. Following mutagenesis of the nucleic acid, the encoded protein can be expressed by any recombinant technology known in the art and the activity of the protein can be determined.

In one embodiment, a mutant RISKMARKER protein can be assayed for (1) the ability to form protein:protein interactions with other RISKMARKER proteins, other cell-surface proteins, or biologically active portions thereof, (2) complex formation between a mutant RISKMARKER protein and a RISKMARKER ligand; (3) the ability of a mutant RISKMARKER protein to bind to an intracellular target protein or biologically active portion thereof; (*e.g.*, avidin proteins); (4) the ability to bind ATP; or (5) the ability to specifically bind a RISKMARKER protein antibody.

In other embodiment, the fragment of the complementary polynucleotide sequence described in claim 1 wherein the fragment of the complementary polynucleotide sequence hybridizes to the first sequence.

In other specific embodiments, the nucleic acid is RNA or DNA. The fragment or the fragment of the complementary polynucleotide sequence described in claim 38, wherein the fragment is between about 10 and about 100 nucleotides in length, *e.g.*, between about 10 and about 90 nucleotides in length, or about 10 and about 75 nucleotides in length, about 10 and about 50 bases in length, about 10 and about 40 bases in length, or about 15 and about 30 bases in length.

**ANTI-SENSE**

Another aspect of the invention pertains to isolated antisense nucleic acid molecules that are hybridizable to or complementary to the nucleic acid molecule comprising the nucleotide sequence of a RISKMARKER or INJURYMARKER sequence or fragments, analogs or derivatives thereof. An "antisense" nucleic acid comprises a nucleotide sequence that is complementary to a "sense" nucleic acid encoding a protein, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. In specific aspects, antisense nucleic acid molecules are provided that comprise a sequence complementary to at least about 10, 25, 50, 100, 250 or 500 nucleotides or an entire RISKMARKER or INJURYMARKER coding strand, or to only a portion thereof. Nucleic acid molecules encoding fragments, homologs, derivatives and analogs of a RISKMARKER or INJURYMARKER protein, or antisense nucleic acids complementary to a nucleic acid comprising a RISKMARKER or INJURYMARKER nucleic acid sequence are additionally provided.

In one embodiment, an antisense nucleic acid molecule is antisense to a "coding region" of the coding strand of a nucleotide sequence encoding RISKMARKER or INJURYMARKER. The term "coding region" refers to the region of the nucleotide sequence comprising codons which are translated into amino acid residues. In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding RISKMARKER. The term "noncoding region" refers to 5' and 3' sequences which flank the coding region that are not translated into amino acids (*i.e.*, also referred to as 5' and 3' untranslated regions).

Given the coding strand sequences encoding RISKMARKER or INJURYMARKER disclosed herein, antisense nucleic acids of the invention can be designed according to the rules of Watson and Crick or Hoogsteen base pairing. The antisense nucleic acid molecule can be complementary to the entire coding region of RISKMARKER or INJURYMARKER mRNA, but more preferably is an oligonucleotide that is antisense to only a portion of the coding or noncoding region of RISKMARKER or INJURYMARKER mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of RISKMARKER or INJURYMARKER mRNA. An antisense oligonucleotide can be, for example, about 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides in length. An antisense nucleic acid of the invention can be constructed using chemical synthesis or enzymatic ligation reactions using procedures known in the art. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally

occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used.

Examples of modified nucleotides that can be used to generate the antisense nucleic acid include: 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Alternatively, the antisense nucleic acid can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

The antisense nucleic acid molecules of the invention are typically administered to a subject or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a RISKMARKER or INJURYMARKER protein to thereby inhibit expression of the protein, *e.g.*, by inhibiting transcription and/or translation. The hybridization can be by conventional nucleotide complementarity to form a stable duplex, or, for example, in the case of an antisense nucleic acid molecule that binds to DNA duplexes, through specific interactions in the major groove of the double helix. An example of a route of administration of antisense nucleic acid molecules of the invention includes direct injection at a tissue site. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For example, for systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, *e.g.*, by linking the antisense nucleic acid molecules to peptides or antibodies that bind to cell surface receptors or antigens. The antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. To achieve sufficient

intracellular concentrations of antisense molecules, vector constructs in which the antisense nucleic acid molecule is placed under the control of a strong pol II or pol III promoter are preferred.

In yet another embodiment, the antisense nucleic acid molecule of the invention is an α-anomeric nucleic acid molecule. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual α-units, the strands run parallel to each other (Gaultier *et al.* (1987) *Nucleic Acids Res* 15: 6625-6641). The antisense nucleic acid molecule can also comprise a 2'-o-methylribonucleotide (Inoue *et al.* (1987) *Nucleic Acids Res* 15: 6131-6148) or a chimeric RNA -DNA analogue (Inoue *et al.* (1987) *FEBS Lett* 215: 327-330).

**RIBOZYMES AND PNA MOIETIES**

In still another embodiment, an antisense nucleic acid of the invention is a ribozyme. Ribozymes are catalytic RNA molecules with ribonuclease activity that are capable of cleaving a single-stranded nucleic acid, such as an mRNA, to which they have a complementary region. Thus, ribozymes (*e.g.*, hammerhead ribozymes (described in Haselhoff and Gerlach (1988) *Nature* 334:585-591)) can be used to catalytically cleave RISKMARKER or INJURYMARKER mRNA transcripts to thereby inhibit translation of RISKMARKER or INJURYMARKER mRNA. A ribozyme having specificity for a RISKMARKER or INJURYMARKER -encoding nucleic acid can be designed based upon the nucleotide sequence of a RISKMARKER or INJURYMARKER DNA disclosed herein. For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a RISKMARKER or INJURYMARKER-encoding mRNA. See, *e.g.*, Cech *et al.* U.S. Pat. No. 4,987,071; and Cech *et al.* U.S. Pat. No. 5,116,742. Alternatively, RISKMARKER or INJURYMARKER mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel *et al.*, (1993) *Science* 261:1411-1418.

Alternatively, RISKMARKER or INJURYMARKER gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of a RISKMARKER or INJURYMARKER nucleic acid (*e.g.*, the RISKMARKER or INJURYMARKER promoter and/or enhancers) to form triple helical structures that prevent transcription of the RISKMARKER or INJURYMARKER gene in target cells. See generally,

Helene. (1991) *Anticancer Drug Des.* 6: 569-84; Helene. *et al.* (1992) *Ann. N.Y. Acad. Sci.* 660:27-36; and Maher (1992) *Bioassays* 14: 807-15.

In various embodiments, the nucleic acids of RISKMARKER or INJURYMARKER can be modified at the base moiety, sugar moiety or phosphate backbone to improve, *e.g.*, the stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of the nucleic acids can be modified to generate peptide nucleic acids (see Hyrup *et al.* (1996) *Bioorg Med Chem* 4: 5-23). As used herein, the terms "peptide nucleic acids" or "PNAs" refer to nucleic acid mimics, *e.g.*, DNA mimics, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of PNAs has been shown to allow for specific hybridization to DNA and RNA under conditions of low ionic strength. The synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described in Hyrup *et al.* (1996) above; Perry-O'Keefe *et al.* (1996) *PNAS* 93: 14670-675.

PNAs of RISKMARKER or INJURYMARKER can be used in therapeutic and diagnostic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, *e.g.*, inducing transcription or translation arrest or inhibiting replication. PNAs of RISKMARKER or INJURYMARKER can also be used, *e.g.*, in the analysis of single base pair mutations in a gene by, *e.g.*, PNA directed PCR clamping; as artificial restriction enzymes when used in combination with other enzymes, *e.g.*, S1 nucleases (Hyrup B. (1996) above); or as probes or primers for DNA sequence and hybridization (Hyrup *et al.* (1996), above; Perry-O'Keefe (1996), above).

In another embodiment, PNAs of RISKMARKER or INJURYMARKER can be modified, *e.g.*, to enhance their stability or cellular uptake, by attaching lipophilic or other helper groups to PNA, by the formation of PNA-DNA chimeras, or by the use of liposomes or other techniques of drug delivery known in the art. For example, PNA-DNA chimeras of RISKMARKER or INJURYMARKER can be generated that may combine the advantageous properties of PNA and DNA. Such chimeras allow DNA recognition enzymes, *e.g.*, RNase H and DNA polymerases, to interact with the DNA portion while the PNA portion would provide high binding affinity and specificity. PNA-DNA chimeras can be linked using linkers of appropriate lengths selected in terms of base stacking, number of bonds between the nucleobases, and orientation (Hyrup (1996) above). The synthesis of PNA-DNA chimeras can be performed as described in Hyrup (1996) above and Finn *et al.* (1996) *Nucl Acids Res* 24: 3357-63. For example, a DNA chain can be synthesized on a solid support using standard phosphoramidite coupling chemistry, and modified nucleoside analogs, *e.g.*,

5'-(4-methoxytrityl)amino-5'-deoxy-thymidine phosphoramidite, can be used between the PNA and the 5' end of DNA (Mag *et al.* (1989) *Nucl Acid Res* 17: 5973-88). PNA monomers are then coupled in a stepwise manner to produce a chimeric molecule with a 5' PNA segment and a 3' DNA segment (Finn *et al.* (1996) above). Alternatively, chimeric molecules can be synthesized with a 5' DNA segment and a 3' PNA segment. See, Petersen *et al.* (1975) *Bioorg Med Chem Lett* 5: 1119-11124.

In other embodiments, the oligonucleotide may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (see, *e.g.*, Letsinger *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:6553-6556; Lemaitre *et al.*, 1987, Proc. *Natl. Acad. Sci.* 84:648-652; PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization triggered cleavage agents (See, *e.g.*, Krol *et al.*, 1988, *BioTechniques* 6:958-976) or intercalating agents. (See, *e.g.*, Zon, 1988, *Pharm. Res.* 5: 539-549). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a peptide, a hybridization triggered cross-linking agent, a transport agent, a hybridization-triggered cleavage agent, etc.

## RISKMARKER AND INJURYMARKER POLYPEPTIDES

One aspect of the invention pertains to isolated RISKMARKER or INJURYMARKER proteins, and biologically active portions thereof, or derivatives, fragments, analogs or homologs thereof. Also provided are polypeptide fragments suitable for use as immunogens to raise anti-RISKMARKER or INJURYMARKER antibodies, *e.g.* antibodies against RISKMARKER 1, or RISKMARKER 6-8. In one embodiment, native RISKMARKER or INJURYMARKER proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment, RISKMARKER or INJURYMARKER proteins are produced by recombinant DNA techniques. Alternative to recombinant expression, a RISKMARKER or INJURYMARKER protein or polypeptide can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the RISKMARKER or INJURYMARKER protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language

"substantially free of cellular material" includes preparations of RISKMARKER or INJURYMARKER protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of RISKMARKER or INJURYMARKER protein having less than about 30% (by dry weight) of non-RISKMARKER or INJURYMARKER protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-RISKMARKER or INJURYMARKER protein, still more preferably less than about 10% of non-RISKMARKER or INJURYMARKER protein, and most preferably less than about 5% non-RISKMARKER or INJURYMARKER protein. When the RISKMARKER or INJURYMARKER protein or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of RISKMARKER or INJURYMARKER protein in which the protein is separated from chemical precursors or other chemicals that are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of RISKMARKER or INJURYMARKER protein having less than about 30% (by dry weight) of chemical precursors or non-RISKMARKER or INJURYMARKER chemicals, more preferably less than about 20% chemical precursors or non-RISKMARKER or INJURYMARKER chemicals, still more preferably less than about 10% chemical precursors or non-RISKMARKER or INJURYMARKER chemicals, and most preferably less than about 5% chemical precursors or non-RISKMARKER or INJURYMARKER chemicals.

Biologically active portions of a RISKMARKER or INJURYMARKER protein include peptides comprising amino acid sequences sufficiently homologous to or derived from the amino acid sequence of the RISKMARKER or INJURYMARKER protein, *e.g.*, the amino acid sequence encoded by a nucleic acid comprising RISKMARKER or INJURYMARKER 1-20 that include fewer amino acids than the full length RISKMARKER or INJURYMARKER proteins, and exhibit at least one activity of a RISKMARKER or INJURYMARKER protein. Typically, biologically active portions comprise a domain or motif with at least one activity of the RISKMARKER or INJURYMARKER protein. A biologically active portion of a

RISKMARKER or INJURYMARKER protein can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

A biologically active portion of a RISKMARKER or INJURYMARKER protein of the present invention may contain at least one of the above-identified domains conserved between the RISKMARKER or INJURYMARKER proteins. An alternative biologically active portion of a RISKMARKER or INJURYMARKER protein may contain at least two of the above-identified domains. Another biologically active portion of a RISKMARKER or INJURYMARKER protein may contain at least three of the above-identified domains. Yet another biologically active portion of a RISKMARKER or INJURYMARKER protein of the present invention may contain at least four of the above-identified domains.

Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the functional activities of a native RISKMARKER or INJURYMARKER protein.

In some embodiments, the RISKMARKER or INJURYMARKER protein is substantially homologous to one of these RISKMARKER or INJURYMARKER proteins and retains its the functional activity, yet differs in amino acid sequence due to natural allelic variation or mutagenesis, as described in detail below.

In specific embodiments, the invention includes an isolated polypeptide comprising an amino acid sequence that is 80% or more identical to the sequence of a polypeptide whose expression is modulated in a mammal to which hepatotoxic agent is administered.

## DETERMINING HOMOLOGY BETWEEN TWO OR MORE SEQUENCES

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (*i.e.*, as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity").

The nucleic acid sequence homology may be determined as the degree of identity between two sequences. The homology may be determined using computer programs known in the art, such as GAP software provided in the GCG program package. See *Needleman and Wunsch* 1970 *J Mol Biol* 48: 443-453. Using GCG GAP software with the following settings for nucleic acid sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the analogous nucleic acid sequences referred to above exhibits a degree of identity preferably of at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99%, with the CDS (encoding) part of a DNA sequence comprising RISKMARKER 1, or RISKMARKER 6-8..

The term "sequence identity" refers to the degree to which two polynucleotide or polypeptide sequences are identical on a residue-by-residue basis over a particular region of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over that region of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, U, or I, in the case of nucleic acids) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the region of comparison *(i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The term "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 80 percent sequence identity, preferably at least 85 percent identity and often 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison region.

## CHIMERIC AND FUSION PROTEINS

The invention also provides RISKMARKER chimeric or fusion proteins. As used herein, an RISKMARKER "chimeric protein" or "fusion protein" comprises an RISKMARKER polypeptide operatively linked to a non-RISKMARKER polypeptide. A "RISKMARKER polypeptide" refers to a polypeptide having an amino acid sequence corresponding to RISKMARKER, whereas a "non-RISKMARKER polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein that is not substantially homologous to the RISKMARKER protein, *e.g.*, a protein that is different from the RISKMARKER protein and that is derived from the same or a different organism. Within an RISKMARKER fusion protein the RISKMARKER polypeptide can correspond to all or a

portion of an RISKMARKER protein. In one embodiment, an RISKMARKER fusion protein comprises at least one biologically active portion of an RISKMARKER protein. In another embodiment, an RISKMARKER fusion protein comprises at least two biologically active portions of an RISKMARKER protein. In yet another embodiment, an RISKMARKER fusion protein comprises at least three biologically active portions of an RISKMARKER protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the RISKMARKER polypeptide and the non-RISKMARKER polypeptide are fused in-frame to each other. The non-RISKMARKER polypeptide can be fused to the N-terminus or C-terminus of the RISKMARKER polypeptide.

For example, in one embodiment an RISKMARKER fusion protein comprises an RISKMARKER domain operably linked to the extracellular domain of a second protein. Such fusion proteins can be further utilized in screening assays for compounds which modulate RISKMARKER activity (such assays are described in detail below).

In yet another embodiment, the fusion protein is a GST-RISKMARKER fusion protein in which the RISKMARKER sequences are fused to the C-terminus of the GST (*i.e.*, glutathione S-transferase) sequences. Such fusion proteins can facilitate the purification of recombinant RISKMARKER, *e.g.* RISKMARKER 1, or RISKMARKER 6-8.

In another embodiment, the fusion protein is an RISKMARKER protein containing a heterologous signal sequence at its N-terminus. For example, a native RISKMARKER signal sequence can be removed and replaced with a signal sequence from another protein. In certain host cells (*e.g.*, mammalian host cells), expression and/or secretion of RISKMARKER can be increased through use of a heterologous signal sequence.

In yet another embodiment, the fusion protein is a RISKMARKER-immunoglobulin fusion protein in which the RISKMARKER sequences comprising one or more domains are fused to sequences derived from a member of the immunoglobulin protein family. The RISKMARKER-immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject to inhibit an interaction between a RISKMARKER ligand and a RISKMARKER protein on the surface of a cell, to thereby suppress RISKMARKER-mediated signal transduction *in vivo*. The RISKMARKER-immunoglobulin fusion proteins can be used to affect the bioavailability of an RISKMARKER cognate ligand. Inhibition of the RISKMARKER ligand/RISKMARKER interaction may be useful therapeutically for both the treatments of proliferative and differentiative disorders, as well as modulating (*e.g.* promoting or inhibiting) cell survival. Moreover, the RISKMARKER

-immunoglobulin fusion proteins of the invention can be used as immunogens to produce anti-RISKMARKER antibodies in a subject, to purify RISKMARKER ligands, and in screening assays to identify molecules that inhibit the interaction of RISKMARKER with a RISKMARKER ligand.

An RISKMARKER chimeric or fusion protein of the invention can be produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, *e.g.*, by employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers that give rise to complementary overhangs between two consecutive gene fragments that can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, Ausubel *et al.* (eds.) Current Protocols in Molecular Biology, John Wiley & Sons, 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). A RISKMARKER -encoding nucleic acid can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the RISKMARKER protein.

## RISKMARKER AND INJURYMARKER AGONISTS AND ANTAGONISTS

The present invention also pertains to variants of the RISKMARKER or INJURYMARKER proteins that function as either RISKMARKER or INJURYMARKER agonists (mimetics) or as RISKMARKER or INJURYMARKER antagonists. Variants of the RISKMARKER or INJURYMARKER protein can be generated by mutagenesis, *e.g.*, discrete point mutation or truncation of the RISKMARKER or INJURYMARKER protein. An agonist of the RISKMARKER or INJURYMARKER protein can retain substantially the same, or a subset of, the biological activities of the naturally occurring form of the RISKMARKER or INJURYMARKER protein. An antagonist of the RISKMARKER or INJURYMARKER protein can inhibit one or more of the activities of the naturally occurring form of the RISKMARKER or INJURYMARKER protein by, for example, competitively binding to a downstream or upstream member of a cellular signaling cascade which includes the RISKMARKER or INJURYMARKER protein. Thus, specific biological effects can be

elicited by treatment with a variant of limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the naturally occurring form of the RISKMARKER or INJURYMARKER proteins.

Variants of the RISKMARKER or INJURYMARKER protein that function as either RISKMARKER or INJURYMARKER agonists (mimetics) or as RISKMARKER or INJURYMARKER antagonists can be identified by screening combinatorial libraries of mutants, *e.g.*, truncation mutants, of the RISKMARKER or INJURYMARKER protein for RISKMARKER or INJURYMARKER protein agonist or antagonist activity. In one embodiment, a variegated library of RISKMARKER or INJURYMARKER variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of RISKMARKER or INJURYMARKER variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential RISKMARKER or INJURYMARKER sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (*e.g.*, for phage display) containing the set of RISKMARKER or INJURYMARKER sequences therein. There are a variety of methods which can be used to produce libraries of potential RISKMARKER or INJURYMARKER variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential RISKMARKER or INJURYMARKER sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, *e.g.*, Narang (1983) *Tetrahedron* 39:3; Itakura *et al.* (1984) *Annu Rev Biochem* 53:323; Itakura *et al.* (1984) *Science* 198:1056; Ike *et al.* (1983) *Nucl Acid Res* 11:477.

**POLYPEPTIDE LIBRARIES**

In addition, libraries of fragments of the RISKMARKER or INJURYMARKER protein coding sequence can be used to generate a variegated population of RISKMARKER or INJURYMARKER fragments for screening and subsequent selection of variants of an RISKMARKER or INJURYMARKER protein. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of a

RISKMARKER or INJURYMARKER coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA that can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the RISKMARKER or INJURYMARKER protein.

Several techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening cDNA libraries for gene products having a selected property. Such techniques are adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of RISKMARKER or INJURYMARKER proteins. The most widely used techniques, which are amenable to high throughput analysis, for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a new technique that enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify RISKMARKER or INJURYMARKER variants (Arkin and Yourvan (1992) PNAS 89:7811-7815; Delgrave *et al.* (1993) Protein Engineering 6:327-331).

## ANTI-RISKMARKER AND ANTI-INJURYMARKER ANTIBODIES

An isolated RISKMARKER or INJURYMARKER protein, or a portion or fragment thereof, can be used as an immunogen to generate antibodies that bind RISKMARKER or INJURYMARKER using standard techniques for polyclonal and monoclonal antibody preparation. The full-length RISKMARKER or INJURYMARKER protein can be used or, alternatively, the invention provides antigenic peptide fragments of RISKMARKER or INJURYMARKER for use as immunogens. The antigenic peptide of RISKMARKER or INJURYMARKER comprises at least 8 amino acid residues of the amino acid sequence encoded by a nucleic acid comprising the nucleic acid sequence shown in RISKMARKER 1-8 and INJURYMARKER 1-10 and encompasses an epitope of RISKMARKER or INJURYMARKER such that an antibody raised against the peptide forms a specific immune complex with RISKMARKER or INJURYMARKER. Preferably, the antigenic peptide

comprises at least 10 amino acid residues, more preferably at least 15 amino acid residues, even more preferably at least 20 amino acid residues, and most preferably at least 30 amino acid residues. Preferred epitopes encompassed by the antigenic peptide are regions of RISKMARKER or INJURYMARKER that are located on the surface of the protein, *e.g.*, hydrophilic regions. As a means for targeting antibody production, hydropathy plots showing regions of hydrophilicity and hydrophobicity may be generated by any method well known in the art, including, for example, the Kyte Doolittle or the Hopp Woods methods, either with or without Fourier transformation. See, *e.g.*, Hopp and Woods, 1981, Proc. Nat. Acad. Sci. USA 78: 3824-3828; Kyte and Doolittle 1982, J. Mol. Biol. 157: 105-142, each incorporated herein by reference in their entirety.

RISKMARKER or INJURYMARKER polypeptides or derivatives, fragments, analogs or homologs thereof, may be utilized as immunogens in the generation of antibodies that immunospecifically-bind these protein components. The term "antibody" as used herein refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds (immunoreacts with) an antigen. Such antibodies include, but are not limited to, polyclonal, monoclonal, chimeric, single chain, $F_{ab}$ and $F_{(ab')2}$ fragments, and an $F_{ab}$ expression library. Various procedures known within the art may be used for the production of polyclonal or monoclonal antibodies to an RISKMARKER or INJURYMARKER protein sequence, *e.g.* RISKMAKER 1 or RISKMAKER 6-8, or derivatives, fragments, analogs or homologs thereof. Some of these proteins are discussed below.

For the production of polyclonal antibodies, various suitable host animals (*e.g.*, rabbit, goat, mouse or other mammal) may be immunized by injection with the native protein, or a synthetic variant thereof, or a derivative of the foregoing. An appropriate immunogenic preparation can contain, for example, recombinantly expressed RISKMARKER or INJURYMARKER protein or a chemically synthesized RISKMARKER or INJURYMARKER polypeptide. The preparation can further include an adjuvant. Various adjuvants used to increase the immunological response include, but are not limited to, Freund's (complete and incomplete), mineral gels (*e.g.*, aluminum hydroxide), surface active substances (*e.g.*, lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, dinitrophenol, etc.), human adjuvants such as *Bacille Calmette-Guerin* and *Corynebacterium parvum*, or similar immunostimulatory agents. If desired, the antibody molecules directed against RISKMARKER or INJURYMARKER can be isolated from the mammal (*e.g.*, from the

blood) and further purified by well known techniques, such as protein A chromatography to obtain the IgG fraction.

The term "monoclonal antibody" or "monoclonal antibody composition", as used herein, refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope of RISKMARKER or INJURYMARKER. A monoclonal antibody composition thus typically displays a single binding affinity for a particular RISKMARKER or INJURYMARKER protein with which it immunoreacts. For preparation of monoclonal antibodies directed towards a particular RISKMARKER or INJURYMARKER protein, or derivatives, fragments, analogs or homologs thereof, any technique that provides for the production of antibody molecules by continuous cell line culture may be utilized. Such techniques include, but are not limited to, the hybridoma technique (see Kohler & Milstein, 1975 *Nature* 256: 495-497); the trioma technique; the human B-cell hybridoma technique (see Kozbor, *et al.*, 1983 *Immunol Today* 4: 72) and the EBV hybridoma technique to produce human monoclonal antibodies (see Cole, *et al.*, 1985 In: Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Human monoclonal antibodies may be utilized in the practice of the present invention and may be produced by using human hybridomas (see Cote, *et al.*, 1983. *Proc Natl Acad Sci USA* 80: 2026-2030) or by transforming human B-cells with Epstein Barr Virus *in vitro* (see Cole, *et al.*, 1985 In: Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96).

According to the invention, techniques can be adapted for the production of single-chain antibodies specific to a RISKMARKER or INJURYMARKER protein (see *e.g.*, U.S. Patent No. 4,946,778). In addition, methods can be adapted for the construction of $F_{ab}$ expression libraries (see *e.g.*, Huse, *et al.*, 1989 *Science* 246: 1275-1281) to allow rapid and effective identification of monoclonal $F_{ab}$ fragments with the desired specificity for a RISKMARKER or INJURYMARKER protein or derivatives, fragments, analogs or homologs thereof. Non-human antibodies can be "humanized" by techniques well known in the art. See *e.g.*, U.S. Patent No. 5,225,539. Antibody fragments that contain the idiotypes to a RISKMARKER or INJURYMARKER protein may be produced by techniques known in the art including, but not limited to: (*i*) an $F_{(ab')2}$ fragment produced by pepsin digestion of an antibody molecule; (*ii*) an $F_{ab}$ fragment generated by reducing the disulfide bridges of an $F_{(ab')2}$ fragment; (*iii*) an $F_{ab}$ fragment generated by the treatment of the antibody molecule with papain and a reducing agent and (*iv*) $F_v$ fragments.

Additionally, recombinant anti-RISKMARKER or INJURYMARKER antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and

non-human portions, which can be made using standard recombinant DNA techniques, are within the scope of the invention. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in PCT International Application No. PCT/US86/02269; European Patent Application No. 184,187; European Patent Application No. 171,496; European Patent Application No. 173,494; PCT International Publication No. WO 86/01533; U.S. Pat. No. 4,816,567; European Patent Application No. 125,023; Better *et al.*(1988) *Science* 240:1041-1043; Liu *et al.* (1987) *PNAS* 84:3439-3443; Liu *et al.* (1987) *J Immunol.* 139:3521-3526; Sun *et al.* (1987) *PNAS* 84:214-218; Nishimura *et al.* (1987) *Cancer Res* 47:999-1005; Wood *et al.* (1985) *Nature* 314:446-449; Shaw *et al.* (1988) *J Natl Cancer Inst.* 80:1553-1559); Morrison(1985) *Science* 229:1202-1207; Oi *et al.* (1986) *BioTechniques* 4:214; U.S. Pat. No. 5,225,539; Jones *et al.* (1986) *Nature* 321:552-525; Verhoeyan *et al.* (1988) *Science* 239:1534; and Beidler *et al.* (1988) *J Immunol* 141:4053-4060.

In one embodiment, methods for the screening of antibodies that possess the desired specificity include, but are not limited to, enzyme-linked immunosorbent assay (ELISA) and other immunologically-mediated techniques known within the art. In a specific embodiment, selection of antibodies that are specific to a particular domain of a RISKMARKER or INJURYMARKER protein is facilitated by generation of hybridomas that bind to the fragment of a RISKMARKER or INJURYMARKER protein possessing such a domain. Antibodies that are specific for one or more domains within a RISKMARKER or INJURYMARKER protein, *e.g.*, domains spanning the above-identified conserved regions of RISKMARKER or INJURYMARKER family proteins, or derivatives, fragments, analogs or homologs thereof, are also provided herein.

Anti-RISKMARKER or anti-INJURYMARKER antibodies may be used in methods known within the art relating to the localization and/or quantitation of a RISKMARKER or INJURYMARKER protein (*e.g.*, for use in measuring levels of the RISKMARKER or INJURYMARKER protein within appropriate physiological samples, for use in diagnostic methods, for use in imaging the protein, and the like). In a given embodiment, antibodies for RISKMARKER or INJURYMARKER proteins, or derivatives, fragments, analogs or homologs thereof, that contain the antibody derived binding domain, are utilized as pharmacologically-active compounds [hereinafter "Therapeutics"].

An anti-RISKMARKER or INJURYMARKER antibody (*e.g.*, monoclonal antibody) can be used to isolate RISKMARKER or INJURYMARKER by standard techniques, such as affinity chromatography or immunoprecipitation. An anti-RISKMARKER or

INJURYMARKER antibody can facilitate the purification of natural RISKMARKER or INJURYMARKER from cells and of recombinantly produced RISKMARKER or INJURYMARKER expressed in host cells. Moreover, an anti-RISKMARKER or INJURYMARKER antibody can be used to detect RISKMARKER or INJURYMARKER protein (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the RISKMARKER or INJURYMARKER protein. Anti-RISKMARKER or INJURYMARKER antibodies can be used diagnostically to monitor protein levels in tissue as part of a clinical testing procedure, *e.g.*, to, for example, determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include $^{125}I$, $^{131}I$, $^{35}S$ or $^{3}H$.

## RISKMARKER RECOMBINANT VECTORS AND HOST CELLS

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid encoding RISKMARKER protein, *e.g.*. RISKMARKER 1, or RISKMARKER 6-8, or derivatives, fragments, analogs or homologs thereof. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a linear or circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein

as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, that is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (*e.g.*, in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (*e.g.*, tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein (*e.g.*, RISKMARKER proteins, mutant forms, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of RISKMARKER in prokaryotic or eukaryotic cells. For example, RISKMARKER can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: (1) to increase expression of recombinant protein; (2) to increase the solubility of the recombinant protein; and (3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith and Johnson (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, Mass.) and pRIT5 (Pharmacia, Piscataway, N.J.) that fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amrann *et al.*, (1988) *Gene* 69:301-315) and pET 11d (Studier *et al.*, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990) 60-89).

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein. See, Gottesman, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, Calif. (1990) 119-128. Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al.*, (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the RISKMARKER expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerevisiae* include pYepSec1 (Baldari, *et al.*, (1987) *EMBO J* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz *et al.*, (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, Calif.), and picZ (InVitrogen Corp, San Diego, Calif.).

Alternatively, RISKMARKER can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect

cells (*e.g.*, SF9 cells) include the pAc series (Smith *et al.* (1983) *Mol Cell Biol* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) *Nature* 329:840) and pMT2PC (Kaufman *et al.* (1987) *EMBO J* 6: 187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells. See, *e.g.*, Chapters 16 and 17 of Sambrook *et al.*, Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv Immunol* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, *e.g.*, the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the -fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner that allows for expression (by transcription of the DNA molecule) of an RNA molecule that is antisense to RISKMARKER mRNA. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen that direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen that direct constitutive, tissue specific or cell type specific

expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub *et al.*, "Antisense RNA as a molecular tool for genetic analysis," Reviews--Trends in Genetics, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but also to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, RISKMARKER protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding

RISKMARKER or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) an RISKMARKER protein, *e.g.* RISKMARKER 1, or RISKMARKER 6-8. Accordingly, the invention further provides methods for producing RISKMARKER protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding RISKMARKER has been introduced) in a suitable medium such that RISKMARKER protein is produced. In another embodiment, the method further comprises isolating RISKMARKER from the medium or the host cell.

## PHARMACEUTICAL COMPOSITIONS

The RISKMARKER nucleic acid molecules, RISKMARKER proteins, and anti-RISKMARKER or anti-INJURYMARKER antibodies (also referred to herein as "active compounds") of the invention, and derivatives, fragments, analogs and homologs thereof, can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein, "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field, which is incorporated herein by reference. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, finger's solutions, dextrose solution, and 5% human serum albumin. Liposomes and non-aqueous vehicles such as fixed oils may also be used. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*,

intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringeability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (*e.g.*, a RISKMARKER protein or anti-RISKMARKER or INJURYMARKER antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are

prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art. The compounds can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable,

biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.* (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

## KITS AND NUCELIC ACID COLLECTIONS FOR IDENTIFYING RISKMARKER AND INJURYMARKER NUCLEIC ACIDS

In another aspect, the invention provides a kit useful for examining hepatotoxicity of agents. The kit can include nucleic acids that detect two or more RISKMARKER or

INJURYMARKER sequences. In preferred embodiments, the kit includes reagents which detect 3, 4, 5, 6, 8, 10, 12, 15, or all of the RISKMARKER or INJURYMARKER nucleic acid sequences.

The invention also includes an isolated plurality of sequences which can identify one or more RISKMARKER or INJURYMARKER responsive nucleic acid sequences. The kit or plurality may include, *e.g.*, sequence homologous to RISKMARKER or INJURYMARKER nucleic acid sequences, or sequences which can specifically identify one or more RISKMARKER or INJURYMARKER nucleic acid sequences.

## OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

**WE CLAIM:**

1. A method of screening a test agent for hepatotoxicity, the method comprising;

(a) providing a test cell population comprising a cell capable of expressing one or more nucleic acid sequences selected from the group consisting of RISKMARKER 1-8 and INJURYMARKER 1-10;

(b) contacting the test cell population with a test agent;

(c) measuring expression of one or more of the nucleic acid sequences in the test cell population;

(d) comparing the expression of the nucleic acid sequences in the test cell population to the expression of the nucleic acid sequences in a reference cell population comprising at least one cell whose exposure status to a hepatotoxic agent is known; and

(e) identifying a difference in expression levels of the RISKMARKER or INJURYMARKER sequences, if present, in the test cell population and reference cell population,

thereby screening said test agent for hepatotoxicity.

2. The method of claim 1, wherein said hepatoxicity comprises idiosyncratic hepatoxicity.

3. The method of claim 2, wherein the method comprises comparing the expression of one or more nucleic acid sequences selected from the group consisting of RISKMARKER 1-8.

4. The method of claim 2, wherein the method comprises comparing the expression of one or more nucleic acid sequences selected from the group consisting of INJURYMARKER 1-10.

5. The method of claim 1, wherein the method comprises comparing the expression of 6 or more of the nucleic acid sequences.

6. The method of claim 1, wherein the expression of the nucleic acid sequences in the test cell population is decreased as compared to the reference cell population.

7. The method of claim 1, wherein the expression of the nucleic acid sequences in the test cell population is increased as compared to the reference cell population.

8. The method of claim 1, wherein the test cell population is provided in vitro.

9. The method of claim 1, wherein the test cell population is provided ex vivo from a mammalian subject.

10. The method of claim 1, wherein the test cell population is provided in vivo in a mammalian subject.

11. The method of claim 1, wherein the test cell population is derived from a human or rodent subject.

12. The method of claim 1, wherein the test cell population includes a hepatocyte.

13. The method of claim 1, wherein said test agent is an idiosyncratic hepatotoxic agent.

14. The method of claim 1, wherein said test agent is a non-steriodal anti-inflammatory drug (NSAID).

15. The method of claim 3, wherein said hepatotoxic agent is a NSAID.

16. The method of claim 15, wherein said NSAID is a NSAID classified as having a low risk of hepatoxicity, and wherein said test agent is identified as having a low risk of hepatoxicity if no qualitative difference in expression levels is identified in step (e).

17. The method of claim 16, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

18 The method of claim 16, wherein said NSAID is selected from the group consisting of Benoxaprofen, Bromfenac, Diclofenac, Phenylbutazone, and Sulindac.

19. The method of claim 18, wherein said NSAID is selected from the group consisting of Benoxaprofen, Phenylbutazone, and Sulindac.

20. The method of claim 15, wherein said NSAID is a NSAID classified as having a very low risk of hepatoxicity, and wherein said test agent is identified as having a very low risk of hepatoxicity if no qualitative difference in expression levels is identified in step (e).

21. The method of claim 20, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

22 The method of claim 20, wherein said NSAID is selected from the group consisting of Etodolac, Fenoprofen, Flurbiprofen, Ibuprofen, Indomethacin, Ketoprofen, Meclofenamate, Mefenamic Acid, Nabumetone, Naproxen, Oxaprozin, Piroxicam, Suprofen, Tenoxicam, Tolmentin, and Zomepirac.

23. The method of claim 22, wherein said NSAID is selected from the group consisting of Flurbiprofen, Oxaprozin, and Tenoxicam.

24. The method of claim 15, wherein said NSAID is a NSAID classified as having an overdose risk of hepatoxicity, and wherein said test agent is identified as having an overdose risk of hepatoxicity if no qualitative difference in expression levels is identified in step (e).

25. The method of claim 24, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

26 The method of claim 25, wherein said NSAID is selected from the group consisting of Acetaminophen, Acetylsalicylic acid, and Phenacetin.

27. The method of claim 4, wherein said hepatotoxic agent is a NSAID.

28. The method of claim 27, wherein said NSAID is a NSAID classified as inducing hepatocellular damage, and wherein said test agent is identified as likely to induce hepatocellular damage if no qualitative difference in expression levels is identified in step (e).

29. The method of claim 28, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

30 The method of claim 27, wherein said NSAID is selected from the group consisting of Acetaminophen, Flurbiprofen, and Ketoprofen.

31. The method of claim 27, wherein said NSAID is a NSAID classified as inducing cholestasis, and wherein said test agent is identified as likely to induce cholestasis if no qualitative difference in expression levels is identified in step (e).

32. The method of claim 31, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

33 The method of claim 30, wherein said NSAID is selected from the group consisting of Benoxaprofen, Nabumetone, and Sulindac.

34. The method of claim 27, wherein said NSAID is a NSAID classified as inducing elevated transaminase level, and wherein said test agent is identified as likely to induce elevated transaminase level if no qualitative difference in expression levels is identified in step (e).

35. The method of claim 34, wherein said difference in expression levels is determined by comparing expression transformation eigenvectors for said test cell and reference cell populations.

36. The method of claim 34, wherein said NSAID is selected from the group consisting of Zomepirac, Mefenamic acid, and Tenoxicam.

37. A method of assessing the hepatotoxicity of a test agent in a subject, the method comprising:

(a) providing from the subject a test cell population comprising a cell capable of expressing one or more nucleic acid sequences selected from the group consisting of RISKMARKER 1-8 and INJURYMARKER 1-10;

(b) contacting the test cell population with a test agent;

(c) measuring expression of one or more of the nucleic acid sequences in the test cell population; and

(d) comparing the expression of the nucleic acid sequences in the test cell population to the expression of the nucleic acid sequences in a reference cell population comprising at least one cell whose exposure status to a hepatotoxic agent is known;

(e) identifying a difference in expression levels of the nucleic acid sequences, if present, in the test cell population and the reference cell population,

thereby assessing the hepatotoxicity of the test agent in the subject.

38. The method of claim 37, wherein said hepatoxicity comprises idiosyncratic hepatoxicity.

39. The method of claim 38, wherein the method comprises comparing the expression of one or more nucleic acid sequences selected from the group consisting of RISKMARKER 1-8.

40. The method of claim 38, wherein the method comprises comparing the expression of one or more nucleic acid sequences selected from the group consisting of INJURYMARKER 1-10.

41. The method of claim 37, wherein the expression of the nucleic acid sequences in the test cell population is increased as compared to the reference cell population.

42. The method of claim 37, wherein the expression of the nucleic acid sequences in the test cell population is increased as compared to the reference cell population.

43. The method of claim 37, wherein said subject is a human or rodent.

44. The method of claim 37, wherein the test cell population is provided ex vivo from said subject.

45. The method of claim 37, wherein the test cell population is provided in vivo from said subject.

46. The method of claim 37, wherein said test agent is a non-steriodal anti-inflammatory drug (NSAID).

47. The method of claim 37, wherein said hepatotoxic agent is a NSAID.

48. An isolated nucleic acid comprising a nucleic acid sequence selected from the group consisting of a RISKMARKER 1 nucleic acid, a RISKMARKER 6-8 nucleic acid, and their complements.

49. A vector comprising the nucleic acid of claim 48.

50. A cell comprising the vector of claim 49.

51. A pharmaceutical composition comprising the nucleic acid of claim 48.

52. A polypeptide encoded by the nucleic acid of claim 48.

53. An antibody which specifically binds to the polypeptide of claim 52.

54. A kit which detects two or more of the nucleic acid sequences selected from the group consisting of RISKMARKER 1, and RISKMARKER 6-8.

55. An array which detects one or more of the nucleic acid selected from the group consisting of RISKMARKER 1, and RISKMARKER 6-8.

56. A plurality of nucleic acid comprising one or more of the nucleic acid selected from the group consisting of RISKMARKER 1, and RISKMARKER 6-8.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
31 May 2001 (31.05.2001)

PCT

(10) International Publication Number
WO 01/38579 A3

(51) International Patent Classification[7]: C12Q 1/68, C12N 5/10, 15/63, C07K 14/47, 16/18, C07H 21/04

(21) International Application Number: PCT/US00/32049

(22) International Filing Date:
21 November 2000 (21.11.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 60/166,923 | 22 November 1999 (22.11.1999) | US |
| 60/183,531 | 18 February 2000 (18.02.2000) | US |
| 09/717,321 | 20 November 2000 (20.11.2000) | US |

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier applications:

| | |
|---|---|
| US | 60/166,923 (CIP) |
| Filed on | 22 November 1999 (22.11.1999) |
| US | 60/183,531 (CIP) |
| Filed on | 18 February 2000 (18.02.2000) |
| US | 09/717,321 (CIP) |
| Filed on | 20 November 2000 (20.11.2000) |

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**
— *with international search report*

(88) Date of publication of the international search report:
14 February 2002

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

WO 01/38579 A3

(54) Title: METHOD OF IDENTIFYING TOXIC AGENTS USING NSAID-INDUCED DIFFERENTIAL GENE EXPRESSION IN LIVER

(57) Abstract: The invention provides methods of identifying toxic agents, e.g., hepatotoxic agents, using differential gene expression. Also provided are methods of predicting the risk level and or injury type of NSAIDs. Also disclosed are novel nucleic acid sequences whose expression is differentially regulated by NSAIDs.

# INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 00/32049

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12N5/10 C12N15/63 C07K14/47 C07K16/18
C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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| A | A. BHATTACHARJEE ET AL.: "Molecular dissection of dimethylnitrosamine (DMN)-induced hepatotoxicity by mRNA differential display." TOXICOLOGY AND APPLIED PHARMACOLOGY, vol. 150, no. 1, May 1998 (1998-05), pages 186-195, XP002174806 New York, NY, USA the whole document --- -/-- | 1-56 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 August 2001 | 27/08/2001 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Nooij, F |

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Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
PCT/US 00/32049

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